Cytokines, Cell Adhesion Molecules And Bladder Cancer Immunotherapy

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Dedication

To my parents,

for giving me that all-important loving start.

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Declaration

I declare that I have composed and written this thesis and that the work described in this thesis is entirely my own and performed by me or by others directly under my supervision, unless otherwise acknowledged.

Andrew M. Jackson

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Publications Arising From These Studies

1. Hawkyard, S.J., James, K., Prescott, S., Jackson, A.M., Ritchie, A.S.W., Smyth, J.F., Chisholm, G.D. (1991) The effects of recombinant human interferon-gamma on a panel of human bladder cancer cell lines. *J. Urol.* **145**: 1078-1081

2. Jackson, A.M., Hawkyard, S.J., Prescott, S., Ritchie, A.W.S., James, K., Chisholm, G.D. (1992) An investigation of factors influencing the *in vitro* induction of LAK activity against a panel of bladder cancer cell lines. *J. Urol.* **147**: 207-211

3. Hawkyard, S.J., Jackson, A.M., Hawkins, R.A., James, K., Smyth, J.F., Chisholm, G.D. (1992) Expression of interferon-γ receptors on bladder cancer cells: does it correlate with biological response? *Urol. Res.* **20**: 229-232

4. Hawkyard, S.J., Jackson, A.M., James, K., Prescott, S., Smyth, J.F., Chisholm, G.D. (1992) The inhibitory effects of interferon-gamma on the growth of bladder cancer cells. *J. Urol.* **147**: 1399-1403

5. Jackson, A.M., Alexandrov, A.B., Prescott, S., James, K., Chisholm, G.D. (1992) Role of adhesion molecules in lymphokine activated killer cell killing of bladder cancer cells: Further evidence for a third ligand for leucocyte function associated antigen-1. *Immunol.* **76**: 286-291

6. Jackson, A.M., Alexandrov, A.B., Prescott, S., James, K., Chisholm, G.D. (1992) Expression of adhesion molecules by bladder cancer cells: Modulation by interferon-gamma and tumour necrosis factor-alpha. *J. Urol.* (In Press)

7. Jackson, A.M., Alexandrov, A.B., James, K. (1992) Cell adhesion molecules in LAK activity against bladder cancer. *ICRF Press.* (In Press)

8. Jackson, A.M., Hawkyard, S.J., Prescott, S., James, K., Chisholm, G.D. (1992) The immunomodulatory effects of urine from patients with superficial bladder cancer receiving intravesical Evans BCG therapy. *Cancer. Immunol. Immunother.* (In Press)

9. Prescott, S., Hawkyard, S.J., Jackson, A.M., James, K., Chisholm, G.D. (1992) Proceedings of Immuntherapie in der Uro-Onkologie conference. (In Press)

In addition, some of the work described in this thesis has been presented at national and international conferences in Edinburgh, Sheffield, London, Essen, Helsinki, and Budapest.

Abstract

The intravesical administration of *Bacillus Calmette Guerin* for the treatment of transitional cell carcinoma of the bladder is the most effective immunotherapy for any solid human malignancy. Despite this awesome accolade relatively little is understood of its mechanisms of action. This study details the *in vitro* interaction between IL-2 activated lymphocytes and tumour cells, the effect of cytokines produced as a result of immunotherapy on tumour cells and the relationship of these finding to the situation *in vivo*.

Bladder cancer cells were not found to be susceptible to NK cell activity but were found to be differentially susceptible to IL-2 activated lymphocytes. No correlation was evident between the histopathological grade of the tumour. The interaction between these cells was observed to involve intimate contact and the tumour cells were found to constitutively express either ICAM-1 or ICAM-2. The expression of these cell adhesion molecules correlated significantly with the sensitivity of the tumour cells to LAK mediated cytolysis.

Following BCG therapy a variety of cytokines including IFNγ and TNFα are detected in the urine. When bladder cancer cells were cultured in the presence of recombinant IFNγ and TNFα an increase in the levels of ICAM-1 expression was observed. The optimal stimulation was found after 24 hours culture with $100Uml^{-1}$ IFNγ, whilst TNFα stimulated to a lesser extent. Culture in the presence of both cytokines was observed to synergistically induce or augment ICAM-1 expression. Following culture with IFNγ, the tumour cells displayed increased susceptibility to LAK activity, this was significantly correlated with increased ICAM-1 expression. The levels of tumour cell response to IFNγ could not be

correlated with either the abundance or affinity of specific receptors as determined by Scatchard analysis. Thus investigations were initiated into the events down-stream of the ligand-receptor interaction.

Monoclonal antibodies to ICAM-1, decreased the sensitivity of tumour cells to LAK activity. However, monoclonal antibodies to LFA-1 (the ligand for ICAM-1) further blocked the action of LAK cells. The LFA-1 dependent, ICAM-1/2 independent activity was suggestive of a further ligand for LFA-1. Using a sensitive flow-cytometric assay the conjugation of effector to target cells was investigated. Conjugation was shown to be energy dependent, requiring the presence of divalent cations. In the absence of Ca^{2+}, Mg^{2+} and Mn^{2+} were found to increase the percentage of target cells which formed conjugates with LAK cells.

Finally the *in vitro* findings were applied to the *in vivo* situation. Urine from patients receiving BCG therapy was found to augment the expression of MHC class II and ICAM-1 molecules on bladder tumour cells. This urine was found to contain both TNFα and IFNγ. Treatment with neutralizing antibodies to IFNγ, but not TNFα, abolished the biological effects of urine. In addition to the presence of cytokines in urine, several patients had detectable levels of soluble ICAM-1 within the first 12 hours following instillation. The significance of this finding remains unclear as ICAM-1 expression has been correlated with both a propensity to metastasize and with increased susceptibility to leucocyte functions.

In conclusion, these investigations dissect some of the molecules involved in the interaction between LAK and bladder tumour cells. Furthermore, using an *in vitro* model of bladder cancer, the control of these molecules by relevant cytokines was delineated. Finally, reference was made to the situation *in vivo* thereby demonstrating relevance to the disease and its immunotherapy.

1.1 Bladder cancer

1.1.1 As a clinical problem

Bladder cancer is a common disease with over 10,000 new cases presenting in the UK *per annum*. Over recent years there has been an increase in the number of new cases of bladder cancer registered in the UK. In 1975 there were 7611 new cases and in 1986 (the latest available figures) there were 9591 (Office of population censuses and surveys.MB1 no. 19 HMSO). The question as to whether this is a true increase in the incidence of the disease, or whether it reflects better diagnosis and documentation, continues to be debated. Regardless, bladder cancer is indisputably a common disease and in 1986 accounted for 5% of all reported malignancies in England and Wales. In Europe, the mean bladder cancer incidence is estimated at 22.9 per 100,000 males and 5.2 per 100,000 females. The general trend at present is an increase in the incidence of bladder cancer (Koroltchouk *et al* 1987). Today, bladder cancer is the eighth most common cancer among males and the eleventh among females, worldwide.

Bladder cancer is a disease of old age, being more common in males than in females, in Caucasians than blacks or Asians, and in urban rather than rural dwellers (Morrison *et al* 1976). This fact probably reflects the increased exposure to industrial promoters and inducers. Incidence varies between countries, and between regions within each country (Thompson *et al* 1990). In Scotland between 1975 and 1980, incidences varied from 3.2/100,000 in Sutherland to 26.9/100,000 in Stirling (Waterhouse *et al*

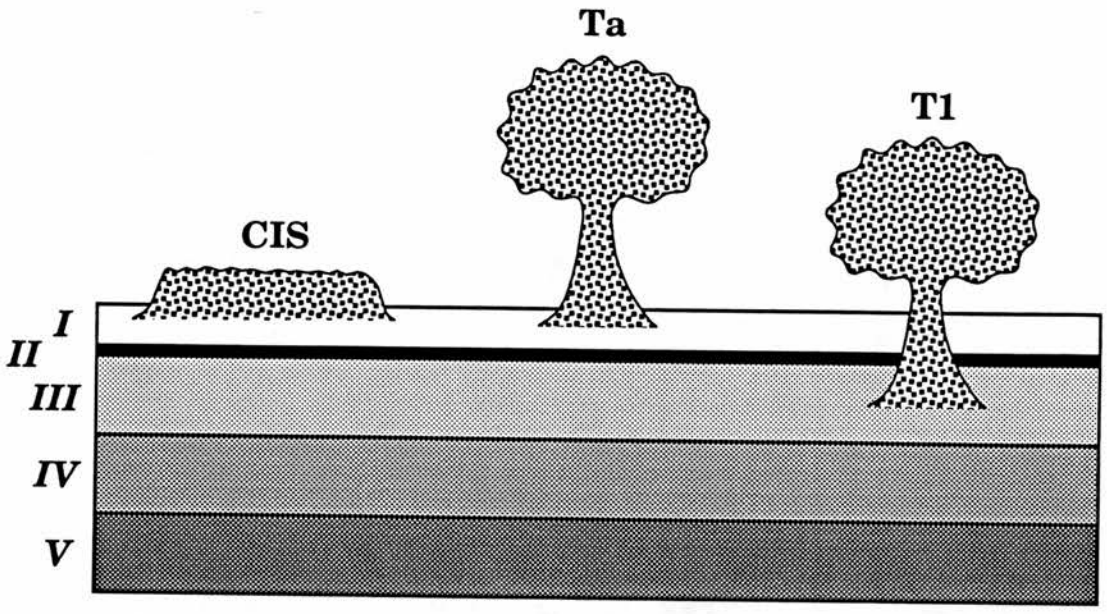
1982. Thompson *et al* 1990).

1.1.2 Pathological classification

In the UK at least 99% of bladder tumours are carcinomas, and more than 90% of these are of the transitional cell type. The majority of patients (>70%) presenting with transitional cell carcinomas have superficial disease (Melicow *et al* 1974). Superficial bladder cancer is defined as disease which does not involve the detrusor muscle, and includes the stages Ta, T1 and carcinoma-in-situ (CIS) (see Figure 1) (Chisholm *et al* 1980). Superficial bladder cancer is not a single disease entity, but rather a spectrum of tumours with markedly different natural histories and prognoses. Transitional cell carcinoma is a malignant diathesis of the whole urothelial tract, a fact which is reflected by its polychronotropic nature, which in turn dictates its management. Histopathologically these tumours are classified into three stages of disease: Cis (carcinoma *in situ*) - a flat, nonpapillary, high-grade carcinoma of the epithelium; Ta - a papillary carcinoma confined to the epithelium, T1 - a tumour invading, but confined to the lamina propria and not involving the superficial muscle layer of the bladder.

A significant proportion (7-22%) of patients with superficial tumours will eventually progress to develop invasive and therefore life threatening bladder cancers (Barnes *et al* 1977. Heney *et al* 1983). Initial incorrect staging will account for a few cases of this apparent disease progression (Lutzeyer *et al* 1982), as problems of inadequate tumour sampling and specimen orientation arise when the whole bladder is not available for histopathological study. Four to twelve percent of patients who present with a superficial bladder cancer will die of the disease

Figure 1. The classification of superficial bladder tumours



The TNM classification of superficial bladder tumours. CIS (carcinoma in situ) -a flat nonpapillary, high grade carcinoma of the bladder epithelium; Ta-a papillary carcinoma confined to the epithelium; T1-a tumour invading, but confined to, the lamina propria and not involving the superficial muscle layer of the bladder. I - urothelium; II - basement membrane; III - lamina propria; IV - superficial muscle layer; V - deep muscle layer of the bladder.

(Pocock *et al* 1982).

The depth of infiltration at which a tumour is no longer considered to be superficial is being reduced as criteria are revised. Originally any tumour infiltrating up to half way through the bladder wall was considered superficial (B1) (Jewett *et al* 1946). At present, superficial tumours are restricted to those not associated with muscle invasion. More recently, tumours which penetrate the lamina propria (T1) are having their superficiality called into question. Many workers have demonstrated the less favourable outlook of T1 as compared with Ta tumours (Abel *et al* 1988a. Abel 1988b). Many T1 tumours are poorly differentiated (G3), and true recurrence rates in patients with such tumours (T1 G3) may be in the order of 60% (Jakse *et al* 1987) while progression has been documented in up to 48% (Heney *et al* 1983). The need for a more aggressive approach to the G3 pT1 tumour is now recognised (Birch *et al* 1989). Of all patients presenting with invasive bladder tumours (T2-4), 50% will have regional and/or distant metastases (Prout *et al* 1979), but in more than half of these the metastases are occult (Culter *et al* 1982).

There are several parameters which have clinical use as prognosticators of disease recurrence and progression in superficial cancer. The stage of a tumour at presentation has some predictive value (Abel *et al* 1988a and b,). In Abel's study, only a low number of patients with Ta tumours had progressed to T1, and none had become invasive. However, 9 of 27 T1 tumours had invaded the muscle layer. Furthermore, the majority of Ta tumours had not developed recurrences at 3 years as compared with 30% patients with T1 tumours. The incidence of progression to invasive disease is greater in those tumours showing least differentiation (Heney *et al* 1983). Such high-grade, poorly differentiated tumours have a correspondingly poor prognosis.

The presence of CIS in urothelial biopsies elsewhere in the bladder at the time of presentation is a poor prognostic sign (Smith *et al* 1983). This fact is perhaps not surprising as CIS is a high grade malignant condition which has an aggressive natural course when it involves significant proportions of the urothelium (Utz *et al* 1980). Other cellular abnormalities in the mucosa adjacent to the primary bladder tumour are of prognostic importance. Althausen observed that most of the patients with superficial bladder cancer who went on to develop invasive disease had had either cellular atypia or CIS surrounding the presenting tumour (Althausen *et al* 1976).

The expression of a variety of receptors for growth factors has been studied on bladder cancer cells. Normal urothelial cells do not express the transferrin receptor other than in the basal layer. However, cancer cells show an increase in the expression of this receptor which correlates with stage and grade (Basar *et al* 1991). Epidermal growth factor (EGF) is also of importance in the control and regulation of epidermal cell growth, and its receptor expression has been studied in bladder cancer (Neal *et al* 1990). Expression of growth factor receptors may offer a means of identifying tumours likely to progress. However, none of these factors have proved to have additional and independent prognostic capabilities for the behaviour of bladder cancer, and tumour stage and grade remain the most important predictors of clinical outcome (Pauwels *et al* 1987).

1.1.3 The aetiology of bladder cancer

The earliest known factor implicated in the development of bladder cancer was the exposure of chemical industry workers to aniline dyes (Rehn *et al* 1895). Since then, an ever increasing number of defined

chemicals have been added to the list which now includes dichlorobenzidine, α-naphthylamine, o-dianisidine and auramine. Workers in the dye, rubber, petroleum and printing industries, where such chemicals are either inhaled, ingested or absorbed through the skin, show an increased risk of bladder tumours (Case *et al* 1954. Glashan *et al* 1981). The development of bladder cancer in patients exposed to industrial carcinogens is a long-term problem, the most important part of which is long term follow-up as the latent period from exposure to onset of disease can be several decades. Many studies from around the world have shown that cigarette smoking increases the risk of developing cancers, including bladder cancer (Miller *et al* 1977).

There is little evidence that any dietary agent, including coffee (Morrison *et al* 1984) and saccharin (Kabat *et al* 1986), significantly increases the likelihood of bladder cancer. Early work suggested tryptophan and its metabolites as causative agents (Dunning *et al* 1950. Rauschenbach *et al* 1963). However, controlled studies looking at urinary tryptophan metabolites have not shown any correlation with bladder cancer (Friedlander and Morrison 1981).

Oliver *et al* have proposed that human pappiloma virus (HPV) may have a role in the induction of bladder cancer in a number of patients (Oliver *et al* 1989). Syrjanen demonstrated a fragment of HPV 16 in 5 of 8 bladder tumours (Syrjanen 1989), but this observation needs to be compared with the incidence of HPV in normal or at least non-neoplastic epithelium to raise it beyond the merely speculative.

The molecular biology of urological tumours has been the subject of considerable attention (Russell *et al* 1990). A number of genes termed proto-oncogenes have been reported to have a central role in the regulation of normal cell growth and differentiation (Buckley 1988). The

products of such genes include nuclear transcription factors, molecules involved in signal transduction of growth factor receptors and in growth factor receptors themselves (Schalken *et al* 1988). It has been demonstrated that, in malignant cells, certain proto-oncogenes can be activated to become oncogenes. Several proto-oncogenes have been to be found activated in human urological malignancies (Malone *et al* 1985). One such proto-oncogene, termed c-erbB-1, encodes the epidermal growth factor (EGF) receptor. EGF has been found to stimulate the growth of a number of bladder cancer cell lines in a dose dependent manner (Messing *et al* 1987). Immunohistochemical studies of EGF-recpetor expression in TCC of the bladder have shown a correlation between expression and an increase in the stage and grade of the tumours (Neal *et al* 1985. Berger *et al* 1987).

1.1.4 Conventional management strategies for bladder cancer

Bladder cancer is an accessible tumour which lends itself to local surveillance and excision. After simple cystoscopic resection of the primary lesion there is a high rate of further bladder tumours developing. Reported rates of recurrence vary between 30% when the presenting tumour is small, single and of low grade, to more than 90% when multiple and of high grade (Soloway *et al* 1988).

Superficial bladder cancer is theoretically curable if the local tumour is completely removed. However, between 4-12% of patients presenting with superficial disease eventually die of bladder cancer (Pocock *et al* 1982). These figures represent those patients whose tumours progress during follow-up. Progression is either defined as a higher grade of recurrent tumour (becoming less differentiated), or as a

more advanced stage of invasion. A drawback of treating patients with sequential courses of intravesical chemotherapies or immunotherapies, is that in patients who have tumours that are destined not to respond valuable time may be lost before cystectomy. Bracken reported on a group of patients who had required cystectomy for uncontrollable superficial disease and found their survival to be comparable with age-matched controls (Bracken *et al* 1981). If every patient with superficial TCC underwent cystectomy, the few would possibly benefit at the expense of the many, for whom cystectomy would represent gross over treatment, and who would suffer the morbidity of cystectomy and urinary diversion. The ideal situation is to be able to select those patients who will progress to life threatening disease before it happens. Our current understanding of bladder cancer is insufficient to predict patients who are unlikely to respond to therapy.

Despite recent advances in the treatment of superficial cancer, the prognosis of invasive bladder cancer remains poor. Patients who have no apparent metastases are offered radical loco-regional treatment with radiotherapy, surgery or a combination of the two. Such treatments will cure approximately half of patients (Quilty *et al* 1986), the remainder will have died at 5 years. This death rate corresponds to the percentage of patients with metastatic disease at presentation (Prout, *et al* 1979). Therefore, it is likely that metastasis occurs prior to radical local treatment. As yet there is no good adjuvant treatment for invasive bladder cancer. The possibilities for adjuvant treatment of micrometastases include adoptive immunotherapy (discussed later). To improve on these figures an effective systemic therapy is required. There is some promise with the newer M-VAC (methotrexate, vinblastine, adriamycin and cisplatin) combination chemotherapeutic regime

(Sternberg *et al* 1988).

1.1.5 Immunotherapeutic strategies for the management of bladder cancer

The success of immunotherapy depends upon the tumour bulk and the immunocompetence of the host. The major potential for its use is as adjuvant treatment for minimal residual or micrometastatic disease which remains after surgery, chemotherapy or radiotherapy. Intravesical therapies (chemotherapy and immunotherapy) allow a relatively large amount of drug to come in contact with the abnormal urothelium, whilst at the same time minimizing systemic toxicity.

Several chronic infective diseases have been noted for their association with low cancer incidence, these include tuberculosis, leprosy and syphilis (Fraenkel 1905. Levin 1910). It was presumably with these observations in mind that therapeutic applications of the tubercle bacillus were investigated in the 1920's by Centanni and Rezzeri (Centanni and Rezzeri 1926). Whilst examining the effect of tubercle bacilli on murine carcinomas they noted that it was important for the bacilli to be viable. The importance of both the viability of the bacilli and the intimate contact with the tumour cells has subsequently been confirmed (Zbar *et al* 1972).

Auguste Calmette and Claude Guerin first developed an avirulent strain of Mycobacterium bovis. *Bacillus Calmette Guerin* (BCG) is an attenuated live vaccine developed originally from this strain. It has been in use as a prophylactic vaccine against tuberculosis for about sixty years, and during the 1950's several immunologists, including Freund, described the immunostimulatory effects it had on the mammalian immune system (Freund 1956).

Local immunotherapy using intravesical *bacillus Calmette Guerin*

now has a major role in the treatment and prophylaxis of superficial bladder cancer (Morales *et al* 1976. Herr *et al* 1985. Badalament *et al* 1987). BCG immunotherapy is of clinical benefit in both the prophylaxis (Martinez-Pineiro *et al* 1990) and treatment of superficial bladder cancer including carcinoma-in-situ (Coplen *et al* 1990), and unlike intravesical chemotherapeutic agents, it decreases the rate of progression to muscle-invasive disease (Herr *et al* 1988). Its most important role would seem to be in reducing the incidence of recurrence and the rate of progression to invasive disease. The efficacy of BCG therapy for superficial bladder cancer has been established throughout the world (Martinez-Pineiro *et al* 1990. Cumming *et al* 1989. DeKernion 1985. Steg 1989. Herr *et al* 1986. Kavoussi *et al* 1988. Sarosdy *et al* 1989. Brosman 1985. Shinka 1990).

As with most therapies, BCG therapy is not without side effects. Lamm *et al* have reviewed the complications of BCG administration in over 2000 patients and described significant side effects in 8% (Lamm *et al* 1992). High fevers (3%) and local irritative symptoms remain the most common problems. Systemic BCG infection with pneumonitis and hepatitis occur rarely (0.7%). Four patient deaths have now been reported in association with the administration of BCG vaccine for superficial bladder cancer (Rawls *et al* 1990. Deresiewicz *et al* 1990). At the present time, it appears that the advantages of intravesical BCG for the treatment and prophylaxis of superficial bladder tumours far outweigh the known disadvantages.

The mechanisms of action of BCG have not been fully elucidated. However, clinical and laboratory studies have provided evidence that the local and systemic immune status is increased in response to BCG. Following intravesical instillation of BCG, an infiltration of immunologically active cells into the bladder wall has been observed

(Prescott *et al* 1992). Detailed characterization of the chronic inflammatory infiltrate using monoclonal antibody probes has been undertaken (El-Demiry *et al* 1987. Prescott *et al* 1989. Böhle *et al* 1990). The major infiltrating cell type in the submucosal tissues was found to be the T-cell, the majority of which were of the CD4+ sub-type. These helper T-cells were immunologically activated as judged by the expression the IL-2 receptor. Macrophages and B cells, the latter occurring mainly in lymphoid follicles, have also been identified; however natural killer (NK) cells were rarely observed.

During the twelve hours following instillation, relatively high concentrations of several cytokines (the relevance of which is discussed in chapter *1.3*) are readily detected in the urine. Interleukin-2 (IL-2), a product of activated T-cells, was detected in the urine of patients treated with intravesical BCG (Ratliff *et al* 1986) but not in the urine of either healthy controls or patients with bacterial cystitis. Subsequently other workers have confirmed this observation (Haaff *et al* 1986. Fleischmann *et al* 1989). Interleukin-1 (IL-1) and tumour-necrosis factor-alpha (TNFα), have also been identified in the urine of patients following BCG administration (Böhle *et al* 1990). Interferon-gamma (IFNγ) has been detected in the post-instillation urine of BCG-treated patients, but not in patients receiving intravesical mitomycin C for superficial bladder cancer (Prescott *et al* 1990). However, some controversy exists over the presence of this cytokine in the urine (A. Böhle, personal communication). The kinetics of cytokine secretion following BCG-treatment may be characteristic of a successful response. Levels rise from undetectable pre-instillation levels and peak values are observed between 2 and 6 hours post instillation. Levels have fallen to pre-instillation values 12 hours later. It would seem that with repeated instillation of BCG, peak levels of

cytokines increase.

Following repeated instillation of BCG into the bladder phenotypic changes have been observed. The urothelium, which does not normally express the products of the MHC class II genes, becomes strongly positive for these antigens (Prescott *et al* 1989). Furthermore, an increase in the level of MHC class I expression has also been reported. Circulating antibodies to BCG are found in the serum of patients treated with intravesical BCG vaccine for superficial bladder carcinoma (Winters *et al* 1981). Prescott (1989) proposed that the possible anti-tumour mechanism of BCG therapy may be via enhanced recognition of the tumour antigenicity through *de novo* HLA-class II expression. This is substantiated by studies of animal models, where induction of HLA-class II antigens on tumour cells enhances their eradication by the host (Bateman *et al* 1991).

If the response against bladder cancer following BCG therapy is specific in nature, then candidate antigens are required. Whether these are BCG or tumour derived has not been determined, however, cross-reactivity between BCG and tumour antigens may exist (Minden *et al* 1976). Repeated exposure to bacillus antigens could theoretically result in sufficient numbers of the reactive T-cells to kill the tumour cells as well as the inciting bacilli. Further evidence for the role of T-cells comes from animal models of bladder cancer. When intravesical BCG is administered to athymic mice hosting the transplantable MBT2 tumour, no anti-tumour effects are observed (Ratliff *et al* 1987). However the anti-tumour response can be restored by an intravenous injection of BCG-sensitised splenocytes.

Following cessation of BCG treatment, the state of immunological alertness in the bladder wall decreases, and by 6 months is virtually non-

existent. Importantly, none of the above phenomena have been observed in the bladders of patients treated with conventional intravesical chemotherapy, thus indicating that intravesical BCG is indeed an immunotherapy.

Other forms of immunotherapy have been investigated as possible treatments for superficial bladder cancer, but have not had the same success as BCG vaccine and have largely been abandoned in clinical practice. Recently, a report has emerged using lymphokine activated killer cells for the therapy of invasive bladder cancer, however no clinical responses were observed (Hermann *et al* 1992). This data will be discussed in chapter *1.4*.

1.2 The host/tumour relationship

1.2.1 Normal cellular control

The intimate relationship between the tumour and its host is complex and varies between types of tumour, types of host and the environment within which the two co-exist. One may consider the tumour cell to be an invading pathogen, a pathogen which is antigenically very similar to the host. However, it is in the best interest of the host not to allow a tumour to develop and to eradicate existing tumours.

Many tissue structures are in constant flux as they develop, adapt to changes in their environment, or are repaired following injury. Under such circumstances, the carefully regulated process of resorption, remodelling and repair are important. It is vital for cells to be correctly orientated in order to maintain the function of a tissue system (Fleming 1991). Furthermore, when tissue repair is complete, there must be adequate control signals to cause cessation of the growth. The abnormal growth behaviour of malignant tumours are the reflection of complex abnormalities in physiology which result from expression of mutated or viral genes and/or deregulated expression of normal genes. When cells are subjected to chemical carcinogens and to various forms of radiation, structural damage may occur to the chromosomal DNA. Point mutations and chromosomal breaks result which have the potential to cause a loss of normal control if not repaired by the host cells enzyme system. Any breakdown in these mechanisms could lead the cell into a state of disregulation and eventually neoplasia.

1.2.2 The concept of immune surveillance

The theory of immune surveillance against cancer postulates that the immune system plays a pivotal role in resistance to the development of tumours. Initially, immune surveillance was thought to be exclusively associated with T-cell mediated responses. However, further work has clearly shown that these responses are mainly concerned with virally induced tumours and not with tumours which may arise spontaneously or as a result of chemical carcinogenesis (Allison *et al* 1967). In transplant patients receiving Cyclosporin A (an inhibitor of T-cell response) there is a high incidence of EBV associated lymphoma (Calne 1979). EBV infected B cells from such patients can be propagated *in vitro* indefinitely, as cyclosporin A serves to inactivate the T cells (Hanto *et al* 1985).

One of the main lines of evidence for immune surveillance lies in observations that individuals which are immunosuppressed (naturally or artificially) have a higher incidence of tumour occurrence. Immunosuppressed renal transplant patients have an increased incidence of malignancies (Kinlen 1982). Such allograft recipients had mainly received prednisolone and azathioprine, agents designed to immunosuppress the host in order to prevent graft rejection.

1.2.3 Detection of the tumour cell

Fundamental to the concept of immune surveillance is the ability of the host to identify the tumour cell. Therefore, a great deal of effort has been invested in the identification of antigenic determinants which are unique to the tumour cell. Unfortunately, investigations into the expression of unique antigens by tumour cells has not proved fruitful. In rare cases, a virally encoded protein/glycoprotein has been identified on

the surface of the cell (Schreiber *et al* 1988). The existence of foreign antigen can be understood in virally-induced tumours where viral DNA is integrated with the hosts DNA and is transcribed into foreign protein. In such instances the T-cell response may be involved in surveillance. However, rather than tumour specific antigen, some tumours express tumour associated antigen (TAA). Certain tumours express molecules which are normally expressed only during foetal development or in the adult at a low level. The two most thoroughly described antigens are alpha-foetoprotein (AFP), and carcinoembryonic antigen (CEA).

There is no evidence at present that spontaneous, as opposed to chemical- and viral-induced tumours (other than lymphomas and leukaemias), express specific tumour antigens (Baldwin 1966). The classical cytotoxic T-cell is restricted to the recognition of antigen in the context of MHC class I molecules. However, recent evidence has shown the existence of class II restricted T-cells with cytotoxic activities (Lancki *et al* 1991). Furthermore, a unique subset of peripheral T-cells has been described which exhibit spontaneous, MHC-unrestricted cytotoxic function (Thiele and Lipsky 1989). One study demonstrated that mixtures of CD4+ and CD8+ T cells do not develop significant non-specific killing activity when activated with IL-2 and antibodies to CD3 (Geller *et al* 1991). However, following the removal of either the CD4+ or the CD8+ cells from the culture, a significant level of non-specific killing activity was displayed. The exact mechanism of such inhibition remains unclear, however cytokines such as IL-4 and TGFβ have been attributed with activities of suppression of such responses *in vitro* (Kasid *et al* 1988. Kawakami *et al* 1989. Chapman *et al* 1990). However, it is possible that certain tumours are infiltrated with a predominant population of either CD4+ or CD8+ T cells, in which case non-MHC restricted, T cell mediated cytotoxicity could

be exerted.

In addition to the T-cell response several other responses have been postulated to partake in immune surveillance. NK cells are able to lyse a limited number of tumour cells *in vitro* (Serrate *et al* 1982). Most NK cells express CD56 and the low-affinity receptor for immunoglobulin (CD16) (Herberman and Ortaldo 1981). NK cells have been observed in small primary tumours and in implanted tumours (Gerson 1980). Furthermore, in patients with Chediak-Higashi syndrome an increased tumour incidence correlates with depressed NK activity (Dent *et al* 1966). A more recent study investigated the effect of NK cell co-culture with bladder cancer cell lines (Wang *et al* 1991). Although freshly isolated NK cells had little cytolytic potential, increased cytotoxicity against bladder cancer cells was observed following several days of co-culture. It is therefore possible that NK cells within small primary tumours are somehow activated to kill the tumour cells.

Many investigators have suggested that cells of the monocyte-macrophage lineage may play a role in anti-tumour responses. Macrophages have been identified in a variety of primary tumours including bladder cancer (Mantovani 1990. Gauci and Alexander 1975. Prescott *et al* 1992). It is now well established that macrophages display *in vitro* a considerable potential to nonspecifically enhance or suppress the growth of many cell types and to kill tumours cells with some selectivity. However, in order for such functions to be exerted, the macrophage must first become activated (Fidler *et al* 1976). The factors involved in such activation of macrophages are the products of activated T cells and include interferon-gamma, originally termed macrophage activating factor. Activation of cell killing by macrophages is an important function of interferon-gamma, since tumour-cell lysis by

activated macrophages is in all likelihood part of the mechanism of natural resistance to cancer (Le *et al* 1983). Activated macrophages, therefore can kill some tumour cells *in vitro* and elicit tumour-destructive reactions *in vivo*. Such macrophages kill tumour cells by releasing reactive oxygen intermediates and by producing other cytotoxic cytokines such as tumour necrosis factor-alpha (Urban *et al* 1986). Interestingly, interferon-gamma induces the production and release of tumour necrosis factor-alpha by macrophages (Philip and Epstein 1986). Furthermore, macrophages, like NK cells, are able to exert antibody dependent cellular cytotoxicity (ADCC) via the Fc receptor found on their surface. The interferons alpha and beta are also capable of boosting the tumouricidal activity of macrophages. The phagocytosis of tumour cells by macrophages in the peritoneal cavity of mice is enhanced by treatment with these cytokines (Gresser and Bourali 1970). Furthermore, *in vitro* treatment of human monocytes with interferon-alpha induces cytolytic activity against malignant target cells (Dean and Virelizier 1983).

Polymorphonuclear leucocytes (PMN) can be cytotoxic to certain tumour cells. This cytotoxicity is thought to be mediated by ADCC, the generation of free-radicals, and by the release of enzymes toxic to tumour cells (Van Kessel and Verhoef 1990). It is, however, likely that if the concept of immune surveillance is true then the most successful response will be effected by a combination of all the branches of the immune system.

1.2.4 Mechanisms of evasion by the tumour

Another important aspect of immune surveillance is immunological escape by the tumour. Although derived from a single cell, a tumour mass

is not a homogeneous collection of cells. Vast heterogeneity of growth rate and phenotype exists throughout the tumour. A T-cell response against TAA would have only minimal effect if there was heterogeneous antigen expression. Several workers have documented the production of immunosuppressive compounds, such as prostaglandins of the E series, by tumours (Plesica *et al* 1975). Such compounds may serve to render the host in an immunocompromized state. A summary of some of the means by which a tumour may avoid detection by the host immune system is given in Table 1.

1.2.5 The host immune response to bladder tumour

The lining of the urinary tract is constantly subjected to challenge by a variety of infectious and carcinogenic agents. However, despite the high incidence of contamination with bacteria, infection is rare, and urothelial malignancy limited. This implies an efficient defence system in the tract.

Bladder cancer is, for the majority of patients, a disease of the latter decades of life. It is known that the immune response of an individual varies with their age. The proportions of various immune cells in the periphery have been reported to change (Ritchie 1983). Patients who develop cancer at an advanced age are therefore at a disadvantage as their immune system is less well prepared and able to deal with the tumour. In addition to this, several studies have shown depressed humoral and cellular responses in bladder cancer patients (Bean *et al* 1978. Catalona *et al* 1974. Herr 1976. Brosman and Fahey 1987).

Studies with the peripheral blood of bladder cancer patients has revealed a marked decrease in the numbers of lymphocytes present in

Table 1. Mechanisms of escape from immune surveillance

A. Immunological unresponsiveness of the host *eg.*
i) specifc eg. tolerance, genetic
ii) non specific eg. genetic, ageing, iatrogenic

B. Immunological sanctuaries

C. Tumour "sneaking through"

D. Immunoselection

E. Phenotypic modification *eg.*
i) antigenic modulation
ii) antigen shedding
iii) antigen coating

F. Shift in balance between immune helper and suppressor mechanisms *eg.*
i) increase in suppressive activities
ii) decreased helper T cell effects

G. Abrogation of immune response by humoral factors *eg.*
i) antigen/antibody complexes
ii) release of cytokines (*eg.* TGFß)
iii) tumour products (*eg.* PGE)

H. Immune stimulation of tumour growth

A summary of some of the possible mechanisms of escape from immune surveillance. This information is taken from a review by K. James (1983). Since that time, little has changed in the filed of immune surveillance and tumour escape.

patients with advanced bladder cancer when compared to normal controls or even patients with superficial disease (Mukamel *et al* 1982). Lymphocyte numbers in patients with superficial disease were similar to those in control groups. The mixed lymphocyte reaction (MLR) has also been investigated. Herr *et al* demonstrated significantly lower maximal response by the lymphocytes of patients with metastatic disease compared with controls (Herr *et al* 1976a). Although the number of individuals in the study was low, Herr also suggested that the MLR might offer a useful adjunct in the evaluation of the biological potential of superficial bladder cancer in that those patients with a depressed MLR progressed to invasive disease (Herr 1983). These observations are not exclusive to bladder cancer and hold true for malignancies of various origin.

1.2.6 Tumour Infiltrating Cells.

Tumour infiltrating lymphocytes (TIL) are attracted to a vascularized tumour when the tumour cells secrete chemotactic, angiogenic or colony stimulating factors (Evans 1986. Mantovani 1990). Tumour infiltrating immune cells are theoretically of more benefit in tumour eradication than those in the systemic circulation, as they have the opportunity to be locally effective. However, their presence in the tumour does not necessarily imply anti-tumour function. Their presence in bladder tumours suggests a local immune response to the tumour, and their prognostic value has been studied retrospectively. The interpretation of the significance of such infiltrates may be complicated by superimposed infection.

Studies of immune tumour infiltrates claimed that of 230 tumours examined, those showing greatest tumour lymphocytic infiltration had the

longest patient survival (Sarma 1970). The presence of interleukin-2 receptor on the surface of T-cells is a marker of activation which allows a better functional assessment of the infiltrate. Positive correlation has been noted between a favourable response and the presence of activated T-cells within the tumour (Prescott *et al* 1992).

Most leucocytes which infiltrate bladder tumours are T-cells (Oliver 1982). Furthermore, the normal bladder has an infiltrate of mainly cytotoxic T cells throughout the epithelium and lamina propria (El-Demiry *et al* 1986). This would suggest a protective function against infection and perhaps tumour. Prescott analyzed 43 bladder tumours with a range of grades and stages (Prescott *et al* 1992). A quantitative assessment was made of the infiltrates in both stromal and tumour compartments. The infiltrating cells were predominantly found in the stromal compartment and were mainly T-cells and macrophages. Significantly more T-cells (most of which were activated) and macrophages were found in high stage tumours than in Ta tumours, and in G3 as compared with low grade tumours. Although these results suggest an enhanced local cellular immune response in invasive tumours, this had obviously not resulted in tumour eradication.

Tsujihashi and colleagues isolated tumour infiltrating lymphocytes from bladder cancer patients with tumours of various grades and stages, and compared their cytotoxic capability with peripheral blood lymphocytes (Tsujihashi *et al* 1988. Tsujihashi *et al* 1989). T lymphocytes were the predominant cell type in the 2 populations of cells, contributing 44% of TIL's and 66% of PBL's. Generally, NK cells, macrophages, and B cells in bladder tumours were present in relatively small numbers. The spontaneous NK cytotoxicity and LAK activity of the TIL's was low (<5%). Interleukin-2 (IL-2) generated greater cytotoxic activity in the

PBL's than in the TIL's. However, unlike PBL's, TIL's exhibited an apparent specificity for autologous tumour killing, which was higher than that against allogeneic bladder tumour cells. Such data support the theory that the local infiltrates are directed against the bladder tumour.

Workers have investigated the characteristics and cellular distribution of putative antigens on bladder tumours with the aim of developing neoplastic markers for diagnosis, prognosis and therapy (Paulie 1985). With the aid of monoclonal antibody technology, a number of groups have postulated the existence of several TCC-associated molecules on bladder cancer cell lines, several of which appeared specific for high grade tumour cells (Paulie and Perlmann 1987. Chopin *et al* 1984. Young *et al* 1985). From these studies one may conclude that bladder tumours are potentially immunogenic, thereby increasing the likelihood of detection and eradication by the immune system.

Recently, monoclonal antibodies have been employed in the detection of human bladder tumour (Bamias *et al* 1991). The tumour associated monoclonal AUA1, which reacts with most human carcinomas, was labelled with a radioisotope. The labelled monoclonal was instilled intravesically into the bladders of patients who were known or suspected of having bladder cancer for an hour prior to cystoscopy. Cystoscopic biopsies of both normal and tumour tissue showed that AUA1 targeted selectively to the tumour, uptake in normal urothelium being minimal. Furthermore, there was also greater uptake with tumours of higher grade.

In summary, a number of changes in the immune cell function of patients with bladder carcinoma have been observed. A deterioration in the quantity and quality of lymphocyte function appears in those with advanced disease. Evidence for immune function impairment in patients with superficial disease is not strong but may be present. The use of *in*

vitro measurements of immune function has been suggested for the prediction of biological behaviour of superficial disease, but at present inter-assay and intra-assay variables make reproducibility and standardization unreliable.

1.2.7 Evidence against immune surveillance

The immune surveillance concept is by no means universally accepted as several lines of evidence exist which appear to contradict the above findings. For example, nude mice do not have an increased incidence of tumours (Rygaard and Poulsen 1976), and tumours developing in immunocompromized individuals are leukaemias and lymphomas not the spontaneously occurring solid tumours commonly seen in clinical practice. In fact, it has been postulated that the immune system may have enhancing effects on tumour induction and growth (Prehn 1971). According to Prehn's immunostimualtion hypothesis many tumour go through a phase when their growth may be stimulated by immune mechanisms. Such stimulation occurs early in their growth and may be mediated by antibody, lymphocytes, macrophage and NK cells.

1.3 Cytokines

During the past century a growing number of research workers have reported findings of novel functions which could not be attributed to molecules which were characterized. Terms such as "leucocyte ferments" were used to describe the activity of a broth extracted from cultured leucocytes. As technical expertise increased a number of these factors were purified and investigated. A nomenclature was developed which termed each factor according to its first reported function (*eg.* T-cell growth factor [TCGF]). Unfortunately, as the number of known factors increased, and as workers from different groups investigated the same factor, a vastly complex array of terminology and abbreviations has arisen, which has only served to isolate the cytokine biologist from their neighbours. In order to rationalize the terminology several terms evolved which were designed to encompass a variety of factors. The terms "interleukin" and "lymphokine" are two such names. Interleukins were initially thought to act as communication signals between leucocytes. However, the interleukins could not be constrained and are known to act on, and be produced by a wide variety of different cell types.

1.3.1 Cytokines in general

The term cytokine was initially proposed by Cohen and colleagues in 1974 (Cohen *et al* 1974) and literally translates as cell-move. Cytokines are large polypeptides and glycoproteins (M_r 5 -50kDa) which are produced by a wide variety of cell types with the function of local communication between cells, typical actions being autocrine, paracrine but not endocrine. Cytokines mediate their pleiotropic functions by specific cell surface

receptors which characteristically exhibit a high-affinity for their ligand (K_d 10^{-9}-10^{-12}M). Depending on the individual cytokine, the receptor can be bound by either monomeric, dimeric or even oligomeric forms. Cytokines tend to be produced by a variety of cell types which are often not closely related (*eg.* lymphocytes and endothelial cells produce IL-1). One remarkable feature of cytokines is the vast degree of redundancy and ambiguity in their function, with many cytokines sharing similar functions and individual cytokines exerting a superfluity of actions on an array of cells and tissues. However, with the advent of recombinant DNA technologies, and the subsequent study of pure cytokines, it has become evident that the response to a cytokine is non-linear and that cytokines interact on a single cell to give effects of different order or type to single cytokines alone.

1.3.2 Interleukin-1

Honoured amongst cytokines, interleukin-1 (IL-1) has been designated the first interleukin. The term IL-1 covers two related polypeptides (IL-1α and IL-1β) which share 25% homology at the amino acid level. Both species of IL-1 are initially synthesized as 31 kDa precursor molecules which are subsequently proteolytically cleaved to yield a carboxy-terminal peptide of molecular weight 17.5 kDa. Neither IL-1α or β is glycosylated and is thought to be predominantly composed of β-strands. Although the pro-forms of IL-1 have biological activity this is minor when compared to the activities of the mature forms (Jobling *et al* 1988).

The relative amounts of mRNA for the two species of IL-1 in PBMC differ greatly; IL-1β being the predominant form by up to 50 fold (Demczuk

et al 1988) and the two forms would appear to be separately regulated (Turner *et al* 1989).

1.3.2.1 The interleukin-1 receptor

Despite the differences in amino-acid sequence and in transcriptional control, the two species of IL-1 bind to common receptors. Two major IL-1 receptors have been identified, type I and type II (Bomsztyk *et al* 1989. Chizzonite *et al* 1989). The two receptors have different molecular weights of 80 and 68 kDa respectively. The receptors have different cellular distributions; transformed T-cells and fibroblasts having high numbers of type I receptor, and B-cells, neutrophils and bone marrow cells having high numbers of type II receptors (Matsushima *et al* 1986). Recent reports have indicated that the two receptors may bind IL-1α and β with different affinity (Benjamin and Dower 1990. Scapigliati *et al* 1989). Furthermore, there are also studies showing differential biological actions of IL-1α and β (Boraschi *et al* 1990). The IL-1R has been identified as a soluble form in tissue culture supernatants, capable of binding IL-1 (Symons and Duff 1990). The soluble receptor was of the low molecular weight type and would appear to be the result of proteolytic cleavage rather than as a result of alternative splicing of mRNA. More recently, a great deal of interest has been shown in other possible inhibitors of IL-1, namely IL-1 receptor antagonist (IL-1ra). IL-1ra would appear to bind to the IL-1 receptor in a manner similar to IL-1, however, it has no IL-1-like activity (Hannum *et al* 1990). The role of such proteins may be in the tight regulation of the potent actions of IL-1.

1.3.2.2 The biological effects of IL-1

The biological effects of IL-1 are many-fold and diverse. Its effects are manifested throughout the host in all tissues from the CNS to immunocompetent cells. IL-1 is perhaps most well characterized for its T-cell activating properties where it has been shown to induce IL-2 and IL-2R expression (Alter and Bach 1990). Alone, IL-1 is a poor stimulator of T-cell proliferation, however, with antigen a synergistic effect is achieved by inducing IL-2 and IL-2 receptor gene expression (Mizel 1987. Simic and Stosic 1985).

Interleukin-1 appears to be a pivotal cytokine in the control of immune response. This is well illustrated by the fact that production of a vast number of cytokines are induced by IL-1 either alone or in combination with other mediators such as TNFα or IL-6 (Howells *et al* 1988. Ghezzi and Dinarello 1988. Wellicome *et al* 1990. Portillo *et al* 1989).

Various pathological conditions have been associated with either locally or systemically elevated levels of IL-1. In chronic conditions such as rheumatoid arthritis (RA), hepatitis B infection, and HIV infection IL-1 levels have been reported to be increased (Bendtzen 1988). IL-1 mediates the resorption of bone *in vitro* and therefore is thought to be essential in the chronic destruction of the joints of RA patients (Gowen *et al* 1983. Dingle *et al* 1987). Various neoplastic and leukaemic cells have been demonstrated to produce IL-1. Lymph node cells of patients with Hodgkin's disease, PBMC's of those with monocytic leukaemia, and renal cell carcinoma tissue have all been found to secrete IL-1 (Bodel *et al* 1980).

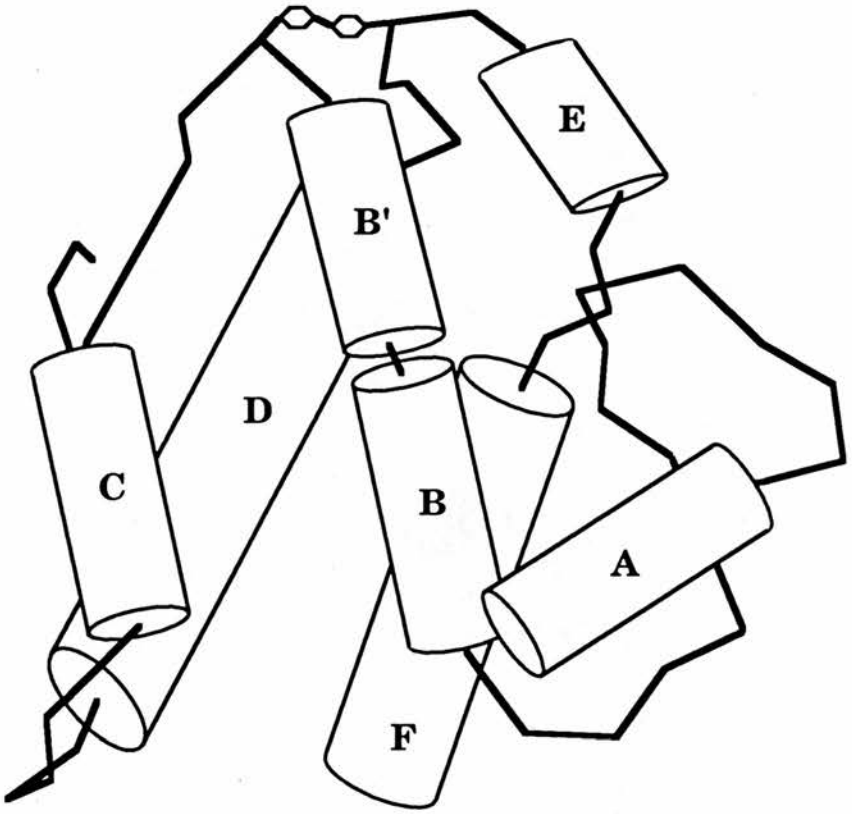
1.3.3 Interleukin-2

Interleukin-2 (IL-2) is a 15.5 kDa glycoprotein which is highly hydrophobic. It is a product of activated T-cells, and recent studies suggest that activated B-cells may also produce IL-2 (Walker *et al* 1988). Mature human IL-2 comprises 133 amino acid residues and shares over 60% homology with murine, rat and bovine IL-2. Like IL-1, IL-2 is secreted as a pro-molecule which requires post-translational modification events to yield a biologically active polypeptide. These events involve cleavage of a 20-residue signal peptide, addition of carbohydrate to the threonine at position 3, and the formation of a disulphide bond between residues 58 and 105 (Robb and Greene 1983). The polypeptide exists as six alpha-helical domains, involving 89 amino acids (Brandhuber *et al* 1987). The three dimensional structure of human IL-2 is shown in Figure 2.

1.3.3.1 The interleukin-2 receptor

The biological effects of IL-2 are mediated by binding to specific receptors. Three forms of IL-2 receptor have been demonstrated to exist giving rise to receptor structures with low, intermediate and high affinities for the ligand. The high affinity receptor (Kd 10-50pM) is composed of two, distinct membrane-associated binding units; the alpha subunit is 55 kDa and the beta subunit is 70-75 kDa (Leonard *et al* 1982. Tsudo *et al* 1987). The IL2-Rα and β subunits can exist as monomers on the cell surface and both are capable of binding IL-2 (IL2-Rα Kd 10,000 pM, IL-2Rβ K_d 1000pM) (Robb 1984). The three different affinities of the IL-2R are a function of the differing binding kinetics of each of the subunits; the alpha

Figure 2. Diagram of the three dimensional structure of human IL-2



The schematized cylinders represent the alpha helical portions of human IL-2. The figure is based on data from Brandhuber *et al* 1987.

unit having a rapid rate of association ($T_{1/2}$=4 seconds), and the beta unit operating far more slowly ($T_{1/2}$=45 minutes) (Wang and Smith 1987. Lowenthal 1987). The high affinity receptor binds IL-2 rapidly and releases it very slowly.

1.3.3.2 The biological effects of interleukin-2

The biological actions of IL-2 include B-cell differentiation, T-cell growth and differentiation, and actions on NK and LAK cells (Splawski *et al* 1990. Grimm *et al* 1982). Quiescent T lymphocytes neither produce IL-2 nor express high-affinity IL-2 receptors (Smith 1980. Robb *et al* 1981). However, following activation with antigen or mitogen, the interaction of IL-2 with its receptor produces rapid clonal expansion of the effector population originally activated by antigen (Robb 1984). Furthermore, IL-2 mediates several of its actions by stimulating the production of a variety of other cytokines including interferon-gamma (IFNγ) and IL-4. (Farrar *et al* 1982. Howard *et al* 1983). B-cells express IL-2R and respond to IL-2, however, receptor expression is 10 fold lower than on activated T-cells (Waldmann *et al* 1984). NK cells also proliferate in response to IL-2, secrete IFNγ and exhibit enhanced cytolytic activity (Ortaldo *et al* 1984).

Interleukin-2 is produced by activated T-cells of the TH1 sub-class (Bottomly 1988). Normal mature T-cell clonal expansion is regulated in an autocrine fashion by IL-2 (Smith 1980) however, abnormal regulation of the IL-2 system is associated with malignant transformation of mature T-cells (Maruyama *et al* 1987. Yamada *et al* 1987).

1.3.4 Interferon-gamma

Interferon-gamma is a glycosylated polypeptide which is the product of a single gene located on human chromosome 12 (Naylor *et al* 1984). It shares no sequence homology or evolutionary relationship with the other interferons. The four exons of the IFNγ gene code for a polypeptide of 166 amino acids, 20 of which constitute a signal peptide (Gray and Goeddel 1982. Taya *et al* 1982). The mature polypeptide contains two potential N-glycosylation sites (positions 28 and 100), as a result of which two different molecular weight forms (20 and 25kDa) of IFNγ have been identified (Yip *et al* 1982. Rinderknecht *et al* 1984). Human IFNγ is acid labile, possibly as a result of the relatively high content of basic residues (Devos *et al* 1982).

1.3.4.1 The interferon-gamma receptor

There is a single species of IFNγ receptor, distinct from the IFNα and β receptors, of molecular weight of around 90 kDa, slight variations of which are probably due to different N-glycosylation patterns (Mao *et al* 1989. Merlin *et al* 1985). The IFNγ receptor is expressed on most nucleated cells (Ucer *et al* 1986). The initial step in the action of IFNγ is binding to its specific receptor (Zoon and Arnheiter 1984). Therefore, the susceptibility of a cell to the biological effects of IFNγ should indicate the presence of receptors on the plasma membrane. Following binding to the receptor the entire ligand-receptor complex is internalized and can be visualized in coated pits with the aid of electron microscopy (Filigueira *et al* 1989). Following endocytosis the ligand is eventually degraded by lysosomal enzymes and the receptor re-cycled to the cell membrane.

A considerable investment has been made into the predictive value of receptor expression (both number and affinity) for the response of a cell

or tissue. Recently, studies have emerged concerning the expression of IFNα receptors by interferon sensitive and resistant urothelial carcinomas (Grups and Bange 1990). Investigators could find no significant difference in either the number of receptors per cell or their relative affinity for ligand which might account for the differential response of the cell. Ucer and colleagues have shown some correlation between the expression of IFNγ receptors and the biological response in a variety of human tumour cell types (Ucer *et al* 1985). In this study the IFNγ sensitivity of inducible cells was dependent on the number of receptors, in that with increasing numbers of receptors present on cells, lower doses of IFNγ were required for the induction of effect. Soluble cytokine receptors have been identified *in vivo*. The soluble receptors for TNF, IL-6 and IFNγ have been identified in normal urine (Englemann *et al* 1989. Novick *et al* 1989). These receptors were shown to prevent the cytotoxic functions of TNFα and IFNγ *in vitro* thus demonstrating a phenomenon that occurs under normal physiological conditions. The presence of these receptors has not been determined in the urine of patients with bladder cancer, however their presence would undoubtedly influence the biological actions of the relevant cytokines.

1.3.4.2 The biological functions of interferon-gamma

In the field of tumour biology, IFNγ has been the subject of considerable attention. Undoubtedly, IFNγ can exert many effects on tumour cells, however these can either be direct (via the specific receptor on the tumour cell) or indirect via other effector mechanisms (some of which are discussed in *1.2.3* and include macrophage activation, increased MHC class I and II stimulation, and effects on T-cells, B-cells, and NK

cells). The growth rate of tumour cells is slowed by IFNγ by causing a prolongation of the cell cycle. However, tumours respond to IFNγ in a heterogeneous manner, ranging from total cytotoxicity, through cytostatic effects to total resistance (Bradley and Ruscetti 1981. Epstein and Marcus 1981. Ludwig *et al* 1983. Balkwill *et al* 1983).

1.3.4.3 Signal transduction by the interferon-gamma receptor

Signalling by the IFNγ receptor after ligand binding has been studied in numerous cell types, and several pathways have been proposed that implicate the involvement of various protein kinases. The activation of protein kinase C (PKC) and Ca^{2+}/calmodulin-dependant protein kinase have been implicated in several studies (Nobukazu *et al* 1990. Koide *et al* 1988. Hamilton *et al* 1985). One such study in glioblastoma cell lines demonstrated that IFNγ inducible class II expression involved the activation of PKC (Nobukazu *et al* 1990). In addition to this, calcium influx appeared to be involved in the DR and DP molecule expressions on T98G cells. These second messengers were found to induce the transcription of DRA and B and DPA and B genes without *de novo* protein synthesis. Further studies on the monocytic U937 cell line showed that following stimulation with IFNγ an increase in intracellular calcium concentration occurred (Klein *et al* 1990), PKC and calmodulin, however, were not found to be involved in the signal transduction.

The functional role of specific signal transduction pathways in the cellular response to exogenous cytokine stimuli can be investigated in several ways. These include measurement of the second messengers generated by the stimulus, determining the cellular response to the stimulus in the presence of inhibitors of specific second messengers, and

determining if direct stimulation of the signal transduction pathway reproduces the cellular response. The understanding of the early events in signal transmission have been greatly facilitated by the advent of pharmacological agents capable of inhibiting or stimulating various intracellular pathways. One such agent is the calcium-ionophore A23187, which has been reported to mimic the effect of IFNγ on the down-regulation of the transferrin receptor and on the priming of macrophages for tumouricidal activity (Weiel *et al* 1985. Johnson and Torres 1985).

The biochemical mechanisms by which IFNγ induces phenotypic and functional changes in a cell have not been fully elucidated. Therefore, depending on the cell type investigated, the IFNγ induced expression of HLA class II molecules involves either a protein kinase C-dependent pathway (Celada and Maki 1991. Koide *et al* 1988. Fan *et al* 1988), or a Ca^{2+}/calmodulin pathway (Klein *et al* 1990. Koide *et al* 1988).

1.3.5 Tumour necrosis factor-alpha

The tumour necrosis factors (TNF) are a two member family of cytokines consisting of the alpha and beta forms. The first TNF to be described was actually TNFβ, originally termed cytotoxic factor and lymphotoxin (Ruddle and Waksman 1968. Granger and Williams 1968). Human TNFβ is a 25kDa protein which shares 35% amino-acid homology with TNFα, a fact which prompted a change in name from lymphotoxin to TNFβ (Li *et al* 1987). Human TNFα was first cloned by Pennica *et al* in 1984 (Pennicia *et al* 1984). Like IL-1, human TNFα is produced as a pro-molecule of 233 residues which is subsequently processed to reveal a mature secreted form of 157 amino acids. The mature 17 kDa form of TNFα is unglycosylated, and recent crystallographic studies have shown

the association of monomers into a compact trimer, and it is in this form that it is thought to associate with its receptor (Jones *et al* 1989).

1.3.5.1 The tumour necrosis factor receptor

As with the receptor for IFNγ, the TNFα receptor is expressed on most cells, with the exception of red blood cells (Aggarwal *et al* 1985. Smith and Baglioni 1989). Two major TNFα receptors have been identified. The smaller of molecular weight 55 kDa and the larger of 75 kDa (Loetscher *et al* 1990. Schall *et al* 1990. Smith *et al* 1990a). The extracellular domains of both receptors are highly similar with six characteristic cysteine residues, however, large differences exist intracellularly indicating differences in signalling pathways. The 75 kDa receptor is expressed by T- and B-cells following activation (Dembric *et al* 1990). However, endothelial cells, the monocytic cell line U937, and NK cells appear to express the two TNF receptors both of which functionally contribute to the actions of TNF. Furthermore, despite the differences in the intracellular domains of the two receptors, both mediate similar functions (Dembric *et al* 1990).

1.3.5.2 The biological effects of tumour necrosis factor

The biological functions of TNFα are diverse and many are held in common with IL-1. TNFα was first shown to be responsible for the chronic wasting condition cachexia found in rabbits infected with *Trypanosoma brucei* and for the dramatic haemorrhagic necrosis of certain types of transplantable tumour (Beutler and Cerami 1989). Both TNFα and TNFβ can act as co-stimulatory signals for activated T-cells, in turn

regulating the growth and differentiation of B-cells. Two important adhesion molecules, endothelial leucocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) are induced on vascular endothelium following stimulation with TNFα or IL-1 (Pober *et al* 1986). The receptor for TNF on T-cells, HLA class II antigen, high affinity IL-2 receptor, and the cytokine IFNγ are induced by stimulation with TNF (Scheurich *et al* 1987. Paul and Ruddle 1988).

In addition to its immunoregulatory role, TNFα has many destructive effects and is thought to be central to several chronic inflammatory disorders. Grau and colleagues showed that TNFα was an essential mediator of murine cerebral malaria induced by *Plasmodium berghei* (Grau *et al* 1987). Normally fatal within 14 days, the neurological manifestations of cerebral malaria could be completely negated with a single injection of anti-TNFα antibody on day 4-7. Other studies with LD_{100} doses of *Escherichia coli* have shown that anti-TNF F(ab')2 fragments when administered 2 hours before the lethal dose can fully protect against shock, vital organ dysfunction, and death (Tracey *et al* 1987). The resorption of cartilage and bone associated with such disease as rheumatoid arthritis, has been found to be a function of TNF and IL-1 (Saklatavala 1986). Furthermore, the production of IL-1 by synovial cells can be reduced by antibodies to TNFα, thus suggesting a possible therapeutic approach for the treatment of the clinical manifestations of RA (Brennan *et al* 1989). In general TNF exerts growth inhibitory effects on some tumour cell lines, but not on untransformed cells (Sugarman *et al* 1985). Some tumour cell lines have been reported to secrete TNFα or β, however, these lines are resistant to the cytotoxic actions (Porter 1991). Elevated TNFα levels have also been implicated as a detrimental factor in neoplastic disease. Systemic TNFα levels have been associated with

cancer in some surveys but not in others (Balkwill *et al* 1987. Socher *et al* 1988). However, one reason for the detrimental effect of TNFα may lie in the systemic metabolic effects rather than in the cellular effects. Many cancer patients die not from the tumour *per se*, but rather from the severe cachexia which frequently accompanies cancer. Antibodies to TNFα have been shown to protect from the antilipogenic and protein wasting effects of TNFα in an animal model (Sherry and Cerami 1988).

More recently, a novel application for TNFα in the treatment of advanced melanoma, sarcoma, and carcinoma of the limbs has been described (Lejeune *et al* 1992). In these clinical trials, limbs were isolated by restriction of the blood supply and TNFα was introduced and allowed to perfuse throughout the limb. Remarkably high rates of complete and partial response were reported, however, the long term response of patients remains to be seen.

1.4 Lymphokine activated killer cells

There are several classical mechanisms of cell mediated killing. Firstly, killing by cytotoxic T-cells involves the recognition of specific processed antigen presented in the context of MHC class-I molecules present on the surface of the target cell. Recognition is achieved by the T-cell receptor (TcR), a heterodimeric structure capable of simultaneously recognising antigen and self-MHC. The CD8 expressing, cytotoxic T-cell recognises endogenous antigens and is therefore largely responsible for the elimination of virally infected target cells. The second form of cytotoxic cell is the natural killer (NK) cell. NK cells are large granular lymphocytes which can kill some transformed, and virally infected target cells *in vitro*. NK cells are characterized by the expression of CD56 (also termed NCAM) and CD16 (Fcγ-III receptor), however, they lack the expression of TcR (Lanier and Phillips 1986. Reynolds and Ortaldo 1987). Thirdly, the macrophage is of central importance in cell mediated cytotoxicity. The macrophage can function both as and APC and as an effector of cytotoxicity.

One decade ago a novel form of cellular cytotoxicity was described. The lymphokine-activated killer (LAK) phenomenon was first reported by Grimm and colleagues (Grimm *et al* 1982). They reported the cytolysis by interleukin-2 activated peripheral blood lymphocytes (PBL) of fresh solid tumour cells which were shown to be resistant to NK mediated lysis. LAK mediated lysis was found to be distinct from other forms of T-cell mediated killing in that it was non-MHC restricted, and did not affect normal cells (Grimm *et al* 1983. Rosenberg and Loetz 1986a). LAK cell were isolated from many patients with solid malignancy, and so it was proposed that LAK cells represented a unique and fundamental cytotoxic effector system

that may play a role in immune surveillance against NK resistant tumour cells, and may be relevant to the adoptive immunotherapy of tumours.

1.4.1 The role of cytokines in the generation of the LAK phenomenon

When non-adherent PBL are stimulated with IL-2 *in vitro* they rapidly proliferate and acquire novel killing capabilities. LAK cells are induced by IL-2 in a dose dependent manner, depending on the source of IL-2 requiring between 1 and 1,000Uml^{-1} (Yamamoto *et al* 1990. Migita *et al* 1989). The kinetics of LAK cell induction are maximal after 6 days of stimulation (Colborn *et al* 1989) and evident following only 3 days of stimulation (Grimm *et al* 1983).

Several other cytokines are able to stimulate the generation of LAK activity in non-adherent PBL and to synergize with IL-2 in the generation of such activity. Synergy between recombinant IL-1 and IL-2 in the generation of LAK activity has been observed, however, a prerequisite was the pretreatment of the cells with IL-1 (Crump *et al* 1989). One possible mechanism by which these two cytokines could act synergistically would be in the induction of the IL-2 receptor β-chain by IL-1. Other mechanisms could include the upregulation of molecules involved in target cell recognition or in the cytolytic process *per se*. When PBL are stimulated with IL-2 they secrete IFNγ (Itoh *et al* 1985. Chapman *et al* 1990). Itoh *et al* hypothesized that IFNγ was involved in the IL-2 induced LAK cytotoxicity and the proliferative response. Interferon-gamma, but not interferon-alpha or beta, has been shown to synergize with low doses (2Uml^{-1}) of IL-2 (Kaufmann *et al* 1991). This data suggests that IL-2 stimulates PBMC to produce IFNγ, which in turn acts as a differentiation signal that may be involved in the IL-2 initiated LAK generation and

proliferative response. Tumour necrosis factor-alpha (TNFα) has also been reported to be produced by PBMC activated with IL-2 and enhance the cytolytic and cytostatic capacity of IL-2 activated killer cells (Chapman *et al* 1990. Matossian-Rogers *et al* 1989. Maghazachi 1990). Such workers showed that TNFα synergizes with low doses of IL-2 or exerted its effects on cells which reacted suboptimally to IL-2 stimulation. Interleukin-7 in the absence of IL-2 induces LAK activity in human PBMC (Stotter *et al* 1991). This novel inducer of LAK activity proved interesting as induction was independent of IL-2 secretion as demonstrated by antibody neutralization.

In contrast to the above observations, cytokines also appear to be involved in the down-regulation of the LAK response. Two cytokines in particular, namely IL-4 and TGFβ, suppress IL-2 induced LAK activity when present, especially during the first 48 hours of stimulation (Kawakami *et al* 1989. Chapman *et al* 1990). Not only was LAK activity inhibited but the PBMC failed to respond with proliferation. Neither IL-4 or TGFβ was found to induce LAK activity when used on their own. Of considerable interest are the findings of Higuchi (Higuchi *et al* 1989). This study compared the actions of IL-4 on freshly isolated PBL and on PBL which had first been activated *in vivo* by IL-2 therapy for advanced cancer. As noted above, IL-4 had no effect of fresh PBL, however, PBL from patients who had received several rounds of IL-2 therapy responded to IL-4 stimulation with an augmentation of their cytolytic capabilities. The positive role of IL-4 in the regulation of LAK activity following *in vivo* activation by IL-2 could also be mimicked *in vitro* thus suggesting that IL-2 activation alters the response of PBL to IL-4. One possible mechanism by which IL-4 may prevent LAK cell generation is by the down-regulation of the high-affinity IL-2 receptor expression (Fernandez-Botran *et al*

1989). However, the changes which reverse the role of IL-4 following IL-2 activation remain unknown. It would seem that IL-2 and IL-4 may act as sequential paracrine co-factors in the induction of precursor cells into LAK effectors. Further studies have shown that the inhibitory effects of IL-4 can be partially reversed if exogenous TNFα or IFNγ are added at the start of culture (Swisher *et al* 1990). The same group also showed that IL-4 decreased the production of TNFα and IFNγ by PBMC (Esner *et al* 1989). Thus one mechanism by which IL-4 may depress LAK activity is by the inhibition of endogenously produced cytokines such as TNFα and IFNγ.

The regulation of LAK activity by cytokines is complex, depending not only on the initial stimulus but also upon the endogenous production of secondary cytokines. Stimulation of PBMC with IL-2 results in the secretion of a plethora of secondary cytokines including both species of IL-1, IL-6, IFNγ, IFNα, TNFα and β, and GM-CSF, some of which may be involved in the LAK phenomenon (Lange *et al* 1991. Dett *et al* 1991) . Furthermore, these cytokines may have direct effects on the target cell if they were secreted in close proximity with the tumour by tumour infiltrating lymphocytes (TIL).

1.4.2 Tumour infiltrating lymphocytes

It has been found that several types of primary tumour frequently exhibit marked lymphocyte infiltration (Saito *et al* 1989. Shimizu *et al* 1991. Belldegrun *et al* 1989. Crannage *et al* 1991). This infiltration of immunocompetent cells into tumour sites might be considered direct evidence of a specific host immune defence mechanism in action (Hiratsuka *et al* 1984). An association may exist between a greater extent of infiltration by lymphocytes into the cancer and fewer metastases and

an improved prognosis (Sarma 1970. Black *et al* 1971). TILs have been identified and isolated from patients with bladder cancer (Tsujihashi *et al* 1988). The TIL were compared with autologous and allogeneic PBL following IL-2 stimulation. There was little spontaneous LAK activity resident in TIL's until stimulated with exogenous IL-2. Unlike the PBL, these TIL exhibited an apparent specificity for bladder tumour; *ie* the cytotoxicity against autologous cells was greater than against allogeneic bladder tumours suggesting some T-cell mediated specific response. Other workers have produced less promising results in that TIL isolated from primary lung cancer patients had significantly lower levels of cytolytic activity than PBL from the same donor (Yano *et al* 1989). The TIL produced low levels of IFNγ, a cytokine thought to be important in the effective generation of LAK activity. A more recent study has shown a correlation between the production of IFNγ during TIL culture and their effectiveness in eradication of established lung tumours in mice, and a lesser relationship between *in vitro* cytotoxicity and clinical response (Barth *et al* 1991). Miyatake and colleagues have transduced the cDNA for IFNγ into a cloned CTL specific for murine glioma, and observed greater efficacy in the immunotherapy of subcutaneous tumours (Miyatake *et al* 1990). More recently, A. Belldegrun presented data concerning gene therapy where the IL-2 gene was inserted into lymphocytes and these subsequently introduced into the tumour (Belldegrun 1992). These studies reveal one possible means of manipulating the TIL for greater clinical benefit, by transducing the cells with cDNA coding for various cytokines including IFNγ and TNFα.

Tumour infiltrating lymphocytes have been demonstrated to produce mRNA for a variety of cytokines following stimulation with IL-2 *in vitro* (Belldegrun *et al* 1989). Transcripts for TNFα, TNFβ, GM-CSF,

and the receptor for IL-2 were detected. It is of interest that no IL-2 mRNA was detected and that unstimulated TIL did not express transcripts for any of the above cytokines. TIL may offer a more successful approach to adoptive immunotherapy than LAK cells. This is supported by the observation that TIL were 50 to 100 times more potent in the killing of autologous tumour cells (Rosenberg *et al* 1986b).

1.4.3 Clinical results obtained using IL-2 and/or LAK cells

The mainstays of current cancer therapy are surgery, radiotherapy and chemotherapy (DeVita 1983). Despite recent advances in early detection of tumours, control of the energy types used in radiotherapy and an increase in the number of chemotherapeutic agents, over 50% of all newly diagnosed cancer patients will die of metastatic cancer (Rosenberg 1988a). Therefore we need a more effective adjuvant therapy and newer effective treatment for systemic disease. Intensive efforts have therefore been directed to the development of novel therapeutic modalities for cancer treatment and the development of biological therapies has been foremost in these efforts.

During the past decade, recent advances in recombinant DNA technology have made available large quantities of highly purified cytokines or biological response modifiers (BRM). One approach to immunotherapy involves the use of such cytokines either directly (for example by *i.v.* administration) or indirectly leading to adoptive immunotherapy. Adoptive cellular immunotherapy (ACI) involves the direct activation of the hosts immune cells *ex vivo* and the reintroduction of such cells into the host to mediate an anti-tumour response, either directly or indirectly. A variety of candidate cells have been investigated for

potential ACI including T-cells, NK cells, monocyte-macrophages and LAK cells. Such *ex vivo* activation of cells has potential advantages in that many effector cells can be generated and therapy does not depend upon a completely intact host immune system.

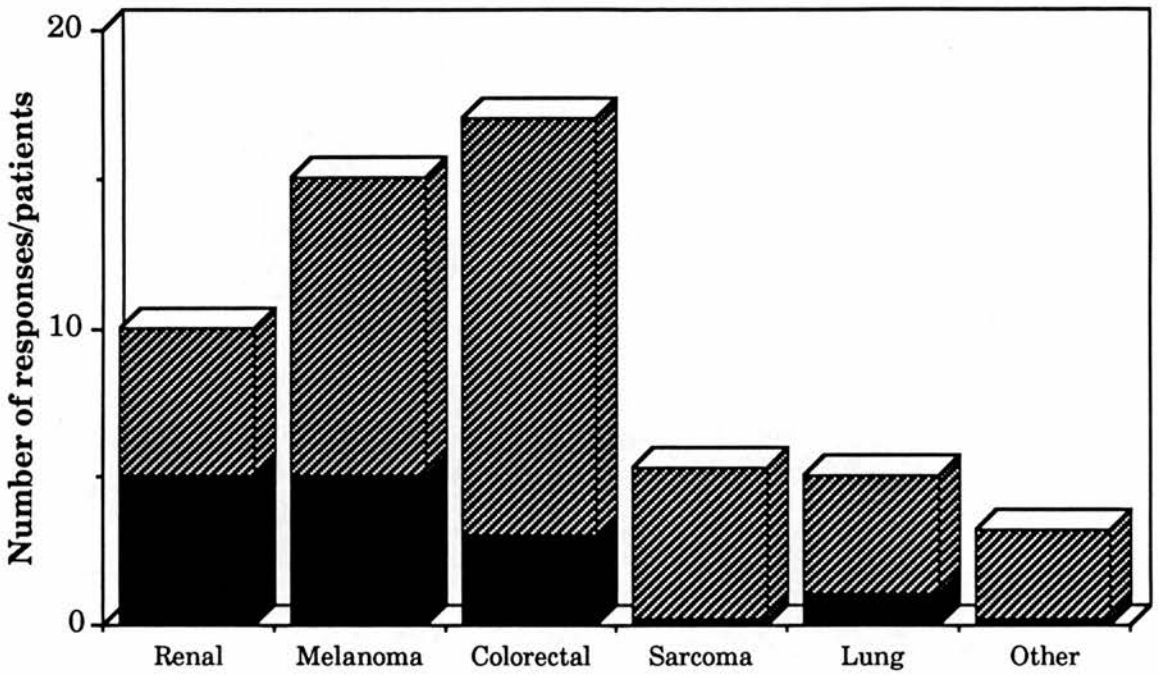
Adoptive cellular immunotherapy was initially undertaken by Rosenberg at the National Cancer institute (Rosenberg *et al* 1985). Such clinical trials in humans involved the single agent use of either IL-2 or LAK cells in escalating doses with the intention of evaluating the toxicity and efficacy of the treatment regimen. However, no response was observed in patients receiving either agent alone. One major problem is the short half-life of IL-2 when systemically administered, typically 2-3 minutes (Donahue and Rosenberg 1983). Therefore, in order to obtain sufficiently high concentrations of IL-2 in the body, extremely large doses of cytokine must be used. Further trials with IL-2 alone showed more promising results with complete remission in 4 and partial response in 9 out of 80 patients treated (Rosenberg 1988b). Although tumour regression can be mediated by IL-2 alone, combined treatment with LAK cells plus IL-2 has yielded greater effect than either treatment alone.

A major problem associated with the systemic administration of high dose IL-2 with or without LAK cells is the high level of toxicity (Stevenson 1989). The majority (>90%) of patients experience nausea, fatigue and fever. Over half of patients have anaemia, abnormal renal function, hypotension, pruritus, diarrhoea, CNS disturbance and eosinophillia. Approximately 20% suffer severe respiratory distress, cardiac arrhythmias, coma, or myocardial ischemia. Furthermore, less than 5% have seizure, myocardial infarction or even death. Although the exact mechanisms of these severe adverse effects has not been clearly elucidated, one important factor seems to be the development of the

capillary leak syndrome leading to fluid shifts in the extravascular tissues.

In addition to the high toxicity associated with IL-2/LAK therapy, the instances of success are noticeably scarce. ACI appears to be most effective in the treatment of malignant melanoma, renal cell carcinoma, non-Hodgkin's lymphoma and colon cancer (Rosenberg *et al* 1987. Fisher *et al* 1987. Dutcher *et al* 1987). Using this regimen an overall response rate of only 25% was achieved for renal cell carcinoma, 21% of melanoma patients and 13% of patients with colorectal carcinoma. Trials with animal models of cancer and LAK cell therapy have shown results which were considerably more encouraging than in clinical trials (Yamaki *et al* 1988. Lafreniere and Rosenberg 1985). Possible reasons for this include the relatively high numbers of transferred effector cells and the ease of administration directly into the lesion rather than systemically. A summary of the responses of various cancer patients is presented in Figure 3.

Figure 3. The response of cancer patients to therapy with LAK cells



A summary of data obtained from Rosenberg et al (1986 Surgery 100: 262.). The responses of cancer patients to adoptive cellular immunotherapy with autologous LAK cells and recombinant IL-2. Shown are the total number of patients (▨), and the number of responders (■). A positive response was defined as a reduction of tumour size by >50%. Other cancers included 1 oesophageal adenocarcinoma, 1 lymphoma, and 1 gastrinoma.

As mentioned in chapter *1.1*, recombinant IL-2 and LAK cell adoptive therapy has recently been used to treat patients with advanced bladder cancer (Hermann *et al* 1992). Substantial changes in the immune system of patients were observed. A migration of leucocytes to the tumours and increased expression of VLA-1 on both peripheral blood mononuclear cells and the endothelial cells of small tumour vessels were seen. Activated T cells (CD25+) and macrophages were seen to infiltrate the tumour, however, few NK cells were noted in the tumour. Systemically, the white blood cell count doubled during therapy and peripheral NK cells were activated by the treatment. Despite the encouraging immunological indicators, no patients responded to the treatment they received and all were affected by varying degrees of toxicity. Recent evidence suggests that following bolus injection of IL-2, a rapid and selective adherence of NK and NK-like lymphocytes to vascular endothelium occurs (Salvo *et al* 1992).

1.4.4 Clinical results obtained with TIL

The emphasis given to tumour infiltrating lymphocytes in recent studies of adoptive immunotherapy stems from the observation made by Rosenberg that TIL were highly effective in causing tumour regression in mice bearing established tumours (Rosenberg *et al* 1986b). Early clinical trials employing IL-2 expanded TIL in adoptive transfers to patients with metastatic cancers refractory to conventional therapy were not especially encouraging (Topalian *et al* 1988. Kradin *et al* 1987). Further human investigations have also been undertaken using autologous TIL which had been expanded *in vitro*. (Kradin *et al* 1989). Response rates of 29% of patients with renal cell carcinoma and 23% of malignant melanoma were

observed, results comparable to those achieved with IL-2/LAK therapy.

From the clinical data it would seem that the early promises of LAK cell investigations *in vitro* have not been realized in the clinical management of cancer. In only a few diseases, notably malignant melanoma and renal cell carcinoma, have any useful clinical results been obtained with LAK therapy. The systemic administration of IL-2 and LAK cells for the treatment of advanced bladder cancer would seem not to be of clinical use. However, this form of therapy may be more efficient when administered into the bladder wall or when used to treat limited and localized disease, rather than disseminated metastatic cancer.

1.5 Cell adhesion molecules

1.5.1 Adhesion in general

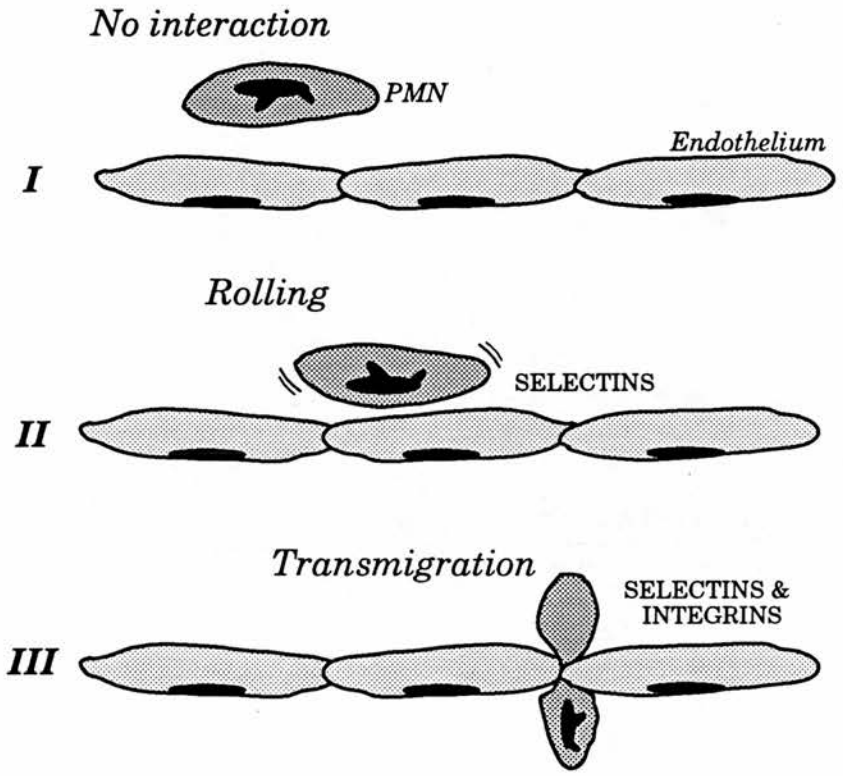
The adhesion of cells to each other and to matrix components is a fundamental requirement for successful communication between cells and structurally correct orientation of individual cells into a tissue (Fleming 1991. Biddlestone and Fleming 1991). These vital functions are achieved by an expanding series of cellular adhesion molecules (Fleming 1990). These molecules may be passive (maintaining adhesion whilst further processes occur) or active in that they can transmit intracellular signals.

1.5.2 Cell-cell adhesion molecules

The leucocytes are highly mobile, presumably so that they can quickly migrate to sites of trauma to deal with repair and invading pathogens. However, the cells must rapidly adhere to the vascular endothelium adjacent to the site of insult in order to transmigrate and gain access. The interaction of circulating cells of the immune system with surrounding tissues, such as endothelium, is initially dependent upon a series of transient adhesion events, controlled by various cell adhesion molecules (see Figure 4). Three superfamilies of adhesion molecules exist: the selectins which appear to interact with carbohydrate containing ligands (*eg* ELAM-1 and GMP-140); the integrins composed of a heterodimer (*eg* LFA-1 and VLA-4); and members of the immunoglobulin superfamily (*eg* ICAM-1, 2, and 3).

The selectins, a family of three members, are implicated in inflammatory and haemostatic response to tissue injury, attracting

Figure 4. A generalized mechanism of recruitment of leucocytes to sites of inflammation



The model depicts the sequential action of selectin and integrin adhesion receptors in the recruitment of leucocytes. I - a suitable leucocyte is attracted to the vascular endothelium adjacent to the site of trauma. II - Using the selectin adhesion molecules the leucocyte rolls along the endothelium until the integrins on its surface are activated. III - following the firm adhesion the leucocyte migrates between adjacent endothelial cells. Finally extravasating cell will terminate its adhesion with the vascular endothelium and migrate to the required site.

neutrophils and other leucocytes to sites of inflammation. They are thought to recognize carbohydrate ligands (Springer and Lasky 1991. Stoolman 1989. Pober and Cotran 1991. Feizi 1991). Two members, ELAM-1 and CD62 are differentially expressed on endothelial cells and platelets in response to a variety of stimuli and recruit T-cells, neutrophils and other myeloid cells to sites of inflammation. Neither ELAM-1 or CD62 is constitutively expressed on endothelial cells or platelets. The expression of ELAM-1 is induced by IL-1β, TNF (both species), IFNγ, and also by bacterial endotoxin (Leeuwenberg *et al* 1990. Bevilacqua *et al* 1987. Bevilacqua *et al* 1989). Cell surface expression is induced after about 4-6 hours. ELAM-1 expression is usually transient, however, in certain chronic inflammatory diseases such as rheumatoid arthritis expression is maintained. The kinetics of CD62 expression are markedly different from ELAM-1 in that newly expressed molecules are readily detected within minutes of stimulation with phorbol ester (Geng *et al* 1990). Mel-14/LAM-1, the third member, is constitutively expressed by some lymphocytes, neutrophils and monocytes (Griffin *et al* 1990. Tedder *et al* 1990. Lewinsohn *et al* 1987). Mel-14/LAM-1 has been demonstrated to play a role in the recruitment of neutrophils to sites of inflammation *in vivo* (Watson *et al* 1991). ELAM-1 has been shown to bind to structures related to the sialyated Lewis-X blood group antigen (SLe^x) (Tiemeyer *et al* 1991). These structures have been identified on the surface of neutrophils and monocytes and termed CD15 (Skubitz *et al* 1983). Furthermore, antibodies to SLe^x have been found to inhibit ELAM-1 mediated adhesion (Walz *et al* 1990). Evidence also exists which suggests that, like ELAM-1, CD62 binds to SLe^x (Larsen *et al* 1990). The relevant ligand/s for Mel-14/LAM-1 have not yet been identified.

The integrins represent a family of cell adhesion receptors expressed

on a wide variety of cell types. They are expressed as transmembrane, non-covalently linked, heterodimers which can be divided into three groups depending upon the expression of the three known available beta chains. Those which share the beta-1 chain are known as very late activation-antigens (VLA-1 - 7), the beta-2 chain as leucocyte-integrins (LFA-1 [leucocyte function associated-antigen-1], Mac-1 and p150,95), and the beta-3 chain as glycoprotein IIb/IIa and vitronectin receptors. The beta 1 and 3 integrins predominantly mediate cell attachment to extracellular matrix, whereas the beta-2 subfamily is mostly involved in cell-cell interactions with the immune system (Arnaout 1990. Mantovani and Dejana 1989). A common feature of some integrins is their ability to adhere to ligands showing a conserved amino acid sequence (arg-gly-asp), the so called RGD sequence. The RGD sequence is present in a number of extracellular proteins such as fibronectin, vitronectin and fibrinogen.

Those adhesion molecules which are members of the immunoglobulin superfamily include such molecules as TcR, MHC classes I and II, CD3, CD4, CD8, NCAM (Neural cell adhesion molecule), and ICAM-1 (intercellular adhesion molecule-1). This group of structures serves many fundamental purposes within the immune system including antigen presentation and recognition.

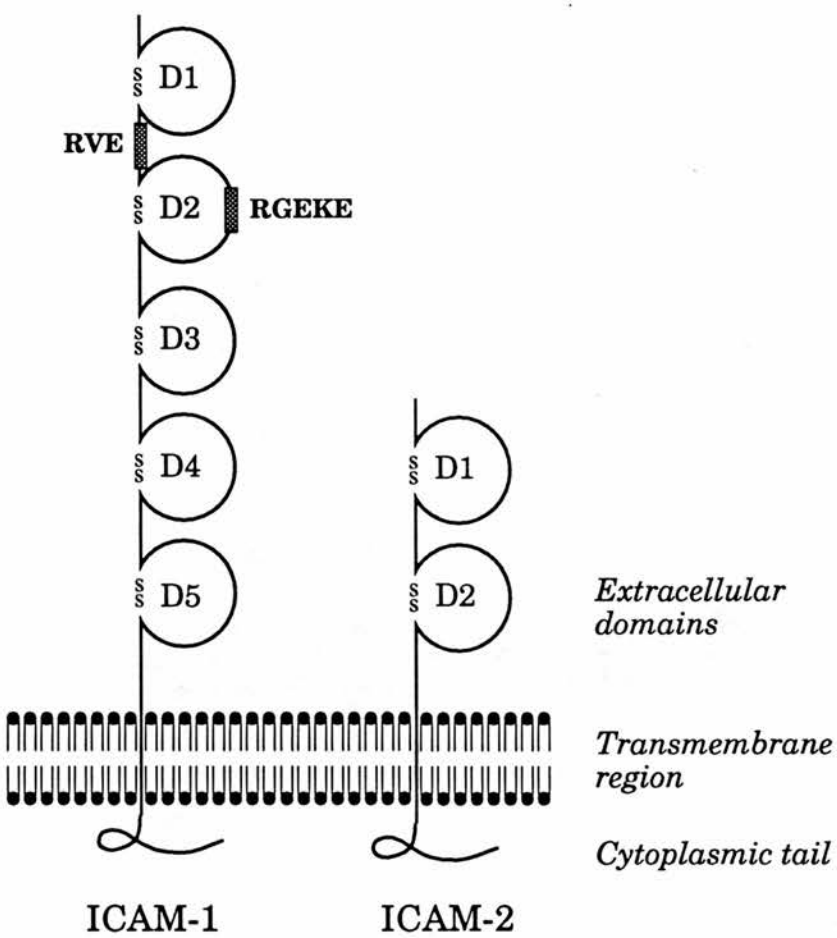
1.5.3 LFA-1 and ICAM-1

The ß2 integrins or leucocyte integrins represent a major sub-group of integrins. Three alpha chains (CD11a, b, and c, M_r 175, 165, and 150 kDa respectively) share a common beta chain (CD18, M_r 95 kDa) giving rise to three distinct adhesion molecules with unique functions; LFA-1 (CD11a/CD18 heterodimer), Mac-1 (CD11b/CD18), and p150-95

(CD11c/CD18). There are three characterized ligands for LFA-1; ICAM-1, -2 and -3 (Rothlein *et al* 1986). ICAM-1 is a monomeric cell surface glycoprotein with a molecular weight of 95kDa. It contains 5 immunoglobulin-like domains, domain two containing an RGEKE (arg-gly-glu-cyc-glu) sequence and an RVE (arg-val-glu) sequence located between the first two domains, however, no RGD sequence exists (see Figure 5) (Marlin and Springer 1987. de Fougerolles *et al* 1991. de Fougerolles and Springer 1992). There is also evidence that ICAM-1 can serve as a ligand for Mac-1 (CD11b) (Smith *et al* 1989). It has been shown that ICAM-1 plays a role in several pathological conditions including airway hyperresponsiveness, renal and cardiac allograft rejection, and the influx of neutrophils in rabbit inflamed lung (Wegner *et al* 1990. Cosimi *et al* 1990. Flavin *et al* 1990. Barton *et al* 1989). ICAM-2 is a 60kDa glycoprotein which has only two immunoglobulin-like domains. The most recently described ligand for LFA-1 is ICAM-3, a highly glycosylated protein of molecular weight 124 kDa (de Fougerolles and Springer 1992).

The cellular and tissue distribution of the three ICAM molecules varies greatly, however one important site of expression unites the three, namely vascular endothelium. ICAM-3 distribution differs markedly from ICAM-1 and -2 in its expression on leucocytes. It is expressed at high levels on resting lymphocytes, monocytes, and neutrophils, whereas ICAM-1 and ICAM-2 were only weakly expressed. Upon activation of lymphocytes with PHA, ICAM-1 expression increases many fold, whereas ICAM-3 expression increases only two to threefold. De Fougerolles and colleagues examined the adhesion of resting and activated lymphocytes to purified LFA-1 (de Fougerolles and Springer 1992). The adherance of resting lymphocytes was shown to be almost entirely ICAM-3 dependent,

Figure 5. Domain diagrams of ICAM-1 and ICAM-2



The single chain polypeptide molecule, ICAM-1, contains 5 immunoglobulin-like domains. The fifth domain lacks a beta-strand. Instead of a RGD-sequence as binding site for LFA-1 the amino acid sequence RVE (arg-val-glu) between the first two domains and RGEKE (arg-gly-glu-cys-glu) within the second domain occur. Also shown is the single chain glycoprotein, ICAM-2. The extracellular domain of ICAM-2 comprises just two immunogloulin-like domains, which exert 34% homology to the first two N-terminal domains of ICAM-1. No RGD sequence exists within the 202 amino acids of ICAM-2. Disulphide bonds are shown (ss).

however, following activation adhesion occurred chiefly via ICAM-1, and to a lesser extent ICAM-3.

The relative affinities of the three ICAMs for LFA-1 also differs markedly, with ICAM-1 having the greatest affinity for its ligand, ICAM-2 and ICAM-3 having similar but lower affinities (de Fougerolles and Springer 1992). The existence of three ligands for LFA-1, the differing tissue distribution, the relative affinities and the varying control of expression by various cytokines indicates specialization for the various aspects of LFA-1 dependent leucocyte interactions. ICAM-1 is basally expressed on endothelium and many epithelial cell types (Springer 1990). The expression of ICAM-1 is strongly induced by a variety of cytokines, including IL-1, IFNγ and TNFα identified with the inflammatory response, and is therefore hypothesized to regulate cell localization and facilitate specific antigen recognition (Altmann *et al* 1989. Siu *et al* 1989). Retinoic acid has also been shown to regulate the expression of ICAM-1 on a variety of human tumour cell lines (Bouillon *et al* 1991). This finding is of particular interest as retinoic acid is known to exert a variety of effects on the growth and differentiation of normal and neoplastic cells (Sporn and Roberts 1983). However, as the maximal induction of ICAM-1 required 4 days it is possible that retinoic acid mediates its action via secondary cytokines. The expression of many other adhesion molecules is regulated by such cytokines. The cytokines IL-1 and TNFα regulate the expression of vascular adhesion molecule-1 (VCAM), the ligand for VLA-4 expressed by leucocytes (Osborn *et al* 1989).

The predominant ligand for LFA-1 on resting endothelium is ICAM-2. This adhesion pathway may have consequences for the recirculation of LFA-1 expressing lymphocytes through endothelium (Hamann *et al* 1988. Mackay *et al* 1990). As mentioned above, ICAM-3 is highly expressed by

leucocytes, thus Springer *et al* (1992) postulated an important role for ICAM-3 in the initiation of an immune response.

1.5.4 The mechanisms of action of LFA-1

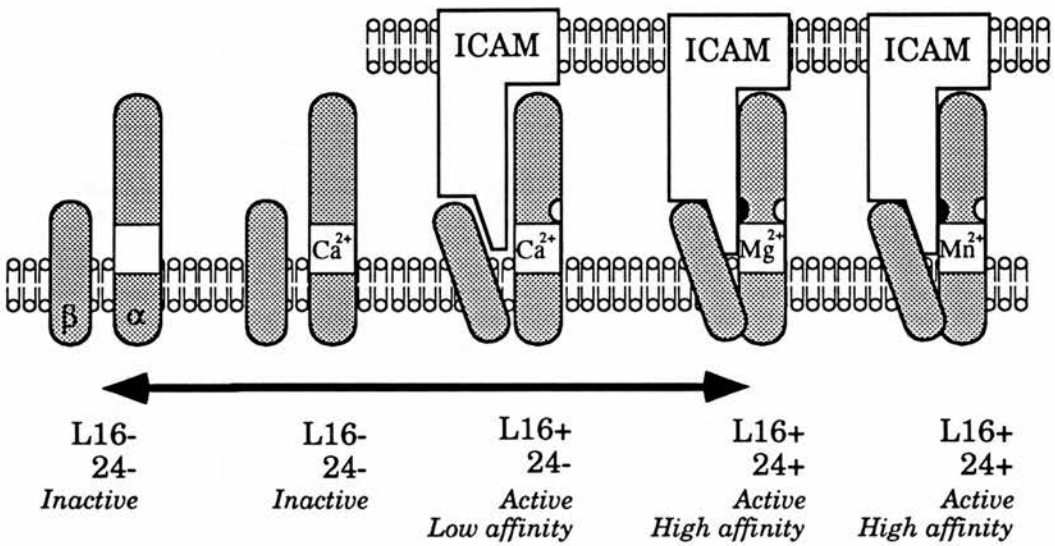
The interaction of LFA-1 with its known ligands is an intricate and highly regulated process. LFA-1, and other integrins, mediate cell adhesion in an energy and temperature dependent process which requires an intact cytoskeleton (Dustin and Springer 1988). Molecular studies of the integrin genes has revealed the presence of three or four potential cation binding sites on the various alpha chains, the significance of which will be discussed. Interestingly, it would appear that expression of LFA-1 alone is not sufficient to permit the interaction with ligand to occur as resting lymphocytes fail to adhere spontaneously yet express high levels of LFA-1. Two main processes would appear to be involved in the activation of LFA-1 from an inactive to an active form. Firstly, the avidity of LFA-1 can be modulated by treatment with agents such as phorbol ester which causes capping of the antigen (Kupfer and Singer 1989). Phorbol myristate acetate (PMA) treatment, therefore, strongly induces LFA-1 dependent cell adhesion (Rothlein and Springer 1986). Following treatment with PMA, the co-distribution of LFA-1 and talin has been reported. The cytoskeletal component of the cell would appear to be important in the function of integrins, as disruption of microfilaments with cytochalasins inhibits leucocyte LFA-1 function (Martz 1987).

The second form of control for functionally active integrin would appear to be independent of avidity. When molecules such as CD2 and CD3 are cross-liked with monoclonal antibodies, intracellular signals are transmitted through the cell, across the membrane to the LFA-1 molecule

resulting in an altered extracellular function (Van Kooyk *et al* 1989). Such activation via CD2 or CD3 is thought to involve the metabolism of inositol phospholipids, thereby activating protein kinase C (PKC), a further mechanism by which PMA is thought to increase LFA-1 dependent functions (Pantaleo *et al* 1987. Imboden and Stobo 1985). PMA has been demonstrated to phosphorylate the cytoplasmic tail of the LFA-1 β-chain (Chatila and Geha 1988). This may induce a conformational change in LFA-1 thereby modifying its affinity for ICAM (Alexander and Cantrell 1989).

The work of two independent research groups lead by Hogg and Figdor have done much to elucidate the possible mechanisms of the activation of LFA-1 from its "off" to its "on" state/s (see Figure 6). Two monoclonal antibodies have served as key tools in the dissection of LFA-1 activation, namely mAb24 and NKI-L16 (Dougherty *et al* 1988. Keizer *et al* 1988). Both monoclonal antibodies identify epitopes on the CD11a molecule, however, the mAb24 also binds to epitopes on the CD11b and c molecules (Dransfield and Hogg 1989. Dransfield *et al* 1990). Binding of the NKI-L16 monoclonal to its epitope on LFA-1 would appear to require the presence of calcium ions. In the absence of calcium, the L16 epitope is not expressed and LFA-1 dependent binding is abolished. Upon binding of the NKI-L16 monoclonal antibody to LFA-1, LFA-1-dependent adhesion is stimulated rather than inhibited. This novel function would appear to be due to a change in conformation and has kinetics similar to those induced by PMA, suggesting the mechanism of action of PMA involves a conformational change (Keizer *et al* 1988). Resting PBL express LFA-1 but virtually lack expression of the L16 epitope (Keizer *et al* 1988. Van Kooyk *et al* 1991). However, following 3-12 hours of stimulation with either PMA or IL-2, the L16 epitope is expressed. Although such cells will now

Figure 6. The possible mechanism of activation of LFA-1



A model of the activation of LFA-1 from inactive to fully active form based on information from Figdor, Hogg and Dransfield. In its "off" state, LFA-1 does not have cations bound to it and fails to express the L16 (◖) or 24 (◗) epitopes. Following binding by calcium the L16 epitope is expressed and interaction with ligand may occur, all be it at a relatively low affinity. Binding by magnesium ions (or possibly manganese) causes conformational changes leading to expression of the 24 epitope and high affinity binding of ICAM.

acquire the ability to aggregate, the expression of the L16 epitope would appear to be a prerequisite but not sufficient for LFA-1 dependent functions. This is well illustrated by the fact that NK cell clones express high levels of L16 but do not spontaneously aggregate (Dustin and Springer 1989). The L16 antibody may maintain the LFA-1 molecule in an active state, thereby increasing LFA-1 dependent adhesion.

In contrast to the L16 antibody, the mAb24 antibody inhibits leucocyte integrin-dependent functions (Dransfield *et al* 1992a). The alpha chain of LFA-1 contains three divalent cation binding sites in domains V, VI and VII. Expression of the mAb24 epitope is Mg^{2+} dependent and parallels receptor activity. Furthermore, the binding of ligand by LFA-1 is dependent upon the presence of divalent cations, in particular Mg^{2+} (Marlin and Springer 1987). Therefore, control of LFA-1 affinity may be achieved by the binding of divalent cations such as calcium and magnesium. The epitope recognized by the mAb24 may be one of the three cation binding sites mentioned previously, as its expression is magnesium dependent. The use of calcium chelating agents such as EGTA have shown that in the presence of Ca^{2+}, the 24 epitope is not expressed (Dransfield and Hogg 1989). In contrast the L16 epitope, once expressed, cannot be reverted to L16 negative. However, at suboptimal concentrations of Mg^{2+}, Ca^{2+} may synergize leading to increased binding (Marlin and Springer 1987). The relative affinities of Mg^{2+} binding by LFA-1 is low when compared to Ca^{2+}, however, 1mM Ca^{2+} does not affect the binding of Mg^{2+} dependent mAb24 (Dransfield *et al* 1992b). Unfortunately, the relationship between L16 and 24 expression has not been fully dissected. It remains to be determined whether the expression of one epitope induces the other.

When the interaction between T-cells and ICAM-1 was studied in the presence and absence of mAb24 a prolongation of the initial adhesion

phase was observed in the presence of the antibody, thus suggesting that mAb24 may act to stabilize the LFA-1/ICAM-1 interaction and prevent the dissociation of these two receptors (Dransfield *et al* 1992a). Therefore, rather than blocking adhesion in the traditional manner of neutralizing antibodies, mAb24 would appear to "lock" the LFA-1 molecule around its ligand.

It would seem therefore that LFA-1 exists in a variety of forms; an inactive form, in which state it is unable to interact with ligand, a fully active form possibly expressing both the L16 and 24 epitopes, and intermediate forms which may be capable of limited interaction with ICAM. The reasons for this remain unclear, however several possibilities exist. The functions of leucocytes which require adhesion include antigen presentation and the delivery of cytotoxic hits. Once initiated or completed, it would be beneficial for the leucocyte to detach. Therefore, some regulatory mechanism is required to mediated adhesion and de-adhesion. As for the function of the intermediate forms of LFA-1 these may serve to mark cells which have previously been activated and are therefore of potential use in an inflammatory response, see for example Haskard *et al* (1986).

In conclusion, the expression of LFA-1 by leucocytes is not sufficient for its function in ligand binding, rather, changes must occur which will modify both its avidity and, perhaps more importantly, its affinity for ICAM. Furthermore, the expression of either the L16 or 24 epitope is not sufficient to initiate adhesion. The interesting observations made with NKI-L16 and mAb24 have provided essential and intricate data, in that NKI-L16 increases the activity of LFA-1 and mAb24 decreases LFA-1 dependent processes, yet does not interfere with the binding of LFA-1 to its ligand. However, as mAb24 has been shown to prevent the de-adhesion

process necessary for re-adhesion to further target cells, this may be the mechanism for the inhibition of LFA-1 dependent processes.

1.5.5 The role of intercellular adhesion molecule-1 in tumour biology

The expression of adhesion molecules which serve as counter receptors for immunologically relevant cells (such as LAK cells) by a tumour may at first glance appear to decrease the chances of survival for that particular tumour. However, recent studies have correlated ICAM-1 expression with an increased propensity for tumour to metastasise (Natalie *et al* 1990). In this study metastatic tumour was found to express higher levels of ICAM-1, and in primary lesion ICAM-1 expression was higher on cells which had undergone malignant transformation. One possible mechanism by which ICAM-1 expression might lead to metastasis is by the interaction with leucocytes lessening the homotypic cohesion between neighbouring tumour cells (Johnson *et al* 1989). Further lines of evidence for a negative role of ICAM-1 in tumour invasion come from studies involving soluble ICAM-1 (Becker *et al* 1991). It has been shown that following stimulation with IFNγ and TNFα, shedding of cell surface ICAM-1 takes place. This soluble ICAM-1 in turn is able to block LFA-1 on leucocytes, thus inhibiting LFA-1 dependent processes. Other studies have correlated increased levels of circulating ICAM-1 with the presence of metastatic cancer, patients with primary lesion alone having significantly lower levels (Tsujiska *et al* 1991).

In contrast to the above data, Vanky *et al* have provided evidence that the metastasis of human tumours is less common when MHC class I antigens and ICAM-1 are highly expressed (Vanky *et al* 1990). Furthermore, a high incidence of metastasis was noted when the levels of

MHC class I were high, and ICAM-1 low. In the light of this evidence it is likely, as for other parameters, that ICAM-1 expression by tumour cells has differing relevance to disease progression depending upon the tumour type, size, and the environment within which it exists.

Recent studies have shown that retinoic acid is able to regulate the expression of ICAM-1 on various tumour cells *in vitro* (Bouillon *et al* 1991). In this study, treatment of four human tumour cell lines (glioma, melanoma, neuroblastoma and teratocarcinoma derived) with 10μM all-*trans* retinoic acid resulted in significant increases in ICAM-1 expression. These increases were comparable with those obtained using $100Uml^{-1}$ IFNγ, however the time course of induction was different. Induction with IFNγ took 24 hours and induction with retinoic acid required 96 hours of continual stimulation. A group of nuclear retinoic acid-inducible trans-acting transcription-enhancing retinoic acid receptors have been discovered (Mangelsdorf *et al* 1990). These receptors serve as transcription factors, mediating response by directly binding to the promoter regions of responsive genes. However, it is unlikely that such elements are involved in the described regulation of ICAM-1 as the kinetics of up-regulation are slow. Rather it is possible that autocrine growth factors are induced which in turn may stimulate ICAM-1 expression. Retinoids (analogues and derivatives of retinol, vitamin A) have been employed in the treatment of bladder cancer with limited success. However, the role of adhesion molecules such as ICAM-1, and its regulation in response to intravesical chemo- and immunotherapy remain to be determined in bladder cancer.

1.6 Current project

The interaction of host immune cells with the tumour cell is vital for the detection and immune mediated eradication of cancer. As has been mentioned, there are several mechanisms which may be utilized by the tumour in order to prevent recognition by surveying cells of the immune system, thus emphasizing the importance of this interaction.

The most successful form of immunotherapy for any solid human malignancy is found in the treatment of superficial bladder cancer. Intravesically administered BCG organisms would appear to cause an acute immunologically based reaction, leading to the infiltration of the bladder wall with T-cells, neutrophils, and monocytes, the production of a variety of cytokines, the modulation of the phenotype of the bladder tumour and the eventual eradication of the cancer. However, despite these observations, little is known concerning the mechanisms of action of BCG. A greater understanding of these processes would perhaps allow the prediction of responders and non-responders, improvement of therapeutic regimens for greater efficacy, and the development of novel immunotherapeutic strategies for the treatment of human cancer. Furthermore, an increased understanding of the role of leucocytes in the anti-tumour response should lead to more effective manipulation of the hosts immune system.

1.6.1. The strategy and experimental approach employed

The aims of these studies were to investigate the role of LAK cells in the mediation of cytolysis of bladder cancer targets, and to elucidate the mechanisms of action of intravesical BCG in the treatment of bladder

cancer. The ideal outcome of this work would be a better understanding of immunotherapy for the treatment of malignant disease and an improved treatment regimen tailored for each patient.

In order to achieve these objectives a tissue culture model of bladder cancer was established using well characterized human transitional cell carcinoma cell lines. These cells were established by Drs. P. Perlmann and J. Masters, during the process of which much was learned of their nature. Several of the cell lines used, in particular RT4, RT112 and MGH-U1 (also termed T24) have been the subject of much attention throughout the world. Although this model is far removed from the situation *in vivo*, the cell lines are representative of their parent tumours and in order to minimize biological drift were used for only a limited number of passages.

The programme of work was as follows:

† *The sensitivity of bladder cancer cells to natural and cytokine enhanced cellular cytotoxicity.* The interaction of LAK cells with bladder cancer cells has not previously been investigated. This area of investigation was a necessary forerunner to further dissection of the mechanism of action of LAK cells. Some of the parameters which were deemed important in the generation of LAK cells were investigated.

† *The expression and modulation of adhesion molecules.* As LAK cell mediated cytotoxicity most probably requires an intimate contact between effector and target cells, the expression of various adhesion molecules on both cell populations was investigated. Earlier studies by Hawkyard *et al* (1991) had demonstrated the phenotypic response of bladder cancer cells to various cytokines. Therefore, the influence of various cytokines (namely IFNγ) on the expression of certain inducible adhesion molecules was investigated.

† *Functional evaluation of the role of adhesion molecules in LAK mediated cytotoxicity against bladder cancer cells.* With the aid of monoclonal antibodies (kindly provided by Dr. T. A. Springer) and a variety of techniques, a functional dissection of the role of cytokines and adhesion molecules in the LAK mediated cytolysis of bladder cancer targets was undertaken.

† *The expression and function of interferon-gamma receptors.* As the cytokine IFNγ had previously been shown to be present in the urine of patients receiving BCG therapy (Prescott *et al* 1990), and to upregulate the expression of HLA class II molecules by bladder cancer cells (Hawkyard *et al* 1991), a study was made of the receptor for IFNγ. Using Scatchard analysis, the dissociation constant (K_d) and the number of receptors per cell was determined for a number of cell lines with differential responses to IFNγ. Furthermore, the kinetics of ligand binding to specific receptor were investigated.

† *The signal transduction of the interferon-gamma receptor.* As differences in the response of cells to IFNγ could not be explained by the receptor status, and were therefore deemed to be due to events down-stream of the receptor/ligand interaction, a pharmacological investigation was made into the nature of the signal transduction mechanism for IFNγ.

† *The relationship of in vitro studies to the situation in vivo.* As this work is concerned with the disease of bladder cancer, it was considered essential to relate some of the *in vitro* findings to the *in vivo* situation. Therefore, the expression of adhesion molecules such as ICAM-1 was studied in tumour biopsy material. The biological effects of urine obtained from patients receiving intravesical BCG therapy were also investigated in the tissue culture model. Furthermore, the secretion of various cytokines into the the urine in response to BCG organisms was

quantified, and a novel parameter, namely the secretion of soluble ICAM-1 into urine, was also investigated.

2 Materials and Methods

2.1 Tissue Culture

All manipulations involving tissue culture were performed in a class II laminar flow safety cabinet (Envair Ltd). Cells were cultured in complete medium (RPMI 1640 (Gibco) supplemented with 5% foetal calf serum (FCS) (Sera-Lab), sodium pyruvate (5mM) and L-glutamine (2mM) (both Gibco), unless otherwise stated. FCS was first heat inactivated by warming to 56°C for 1 hr prior to addition to RPMI medium. In experiments requiring serum free media, HB103 media was used (NEN). The cells were incubated and grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2 Bladder tumour cell lines

Eight human transitional cell carcinoma (TCC) cell lines were used throughout these studies. These cells were the generous gift of Dr. J. Masters of the Institute of Urology, London, Dr. P. Perlmann, Stockholm, Sweden and Dr. J. Boyd, University of Edinburgh, UK. All cell lines were tested mycoplasma free by the Public Health Laboratory Service, Porton Down, UK. The panel of cell lines represents various histopathological tumour grades as detailed in table 2. The cells have proved to be stable and their cytological appearances and growth characteristics have remained representative of the parent tumours. In order to minimize biological drift, cells were used for only a limited number of passages (n=10) before they were discarded and early passage number stocks re-cultured.

For routine culture, cells were seeded at a density of $5x10^4ml^{-1}$ and

Table 2. The grades of the eight cell lines used throughout these studies

Cell Line Name	Parental Tumour Grade	Histpathological Grade
RT4	G1	G1
UMUC3	?	G1
RT112	G2	G2
MGH-U1	G3	G3
EJ18	G3	G3
SD	G3	G3
J82	G3	G3
5637	G3	G3

Eight cell lines were used throughout these studies. They were derived from human transitional cell carcinoma of the bladder. The table gives the grade of the parent tumour and that of the cell line (as determined by a consultant histopathologist). G1 represents well differentiated cells. G2 represents moderately differentiated cells. G3 represents poorly differentiated cells which least resemble the tissue of origin. These cell lines were kindly provided by Dr. J. Masters and by Dr. P. Perlmann.

cultured in antibiotic free complete medium in 25cm^2 tissue culture flasks (Sterelin). Cells were recovered for routine purposes by washing the monolayer with phosphate buffered saline (PBS, Dulbecco A) and then trypsinizing using 1ml trypsin/EDTA solution (0.5 gl^{-1} trypsin and 0.2 gl^{-1} disodium EDTA (Sigma Chemical Co) for every 25cm^2 of tissue culture plastic. Immediately following detachment of the cells, 10ml of complete medium was added to the cell suspension in order to neutralize the trypsin and saturate the EDTA. For some experiments tumour monolayers were released without the aid of trypsin by using 0.02% EDTA solution in PBS for 15 minutes.

2.3 Isolation of peripheral blood mononuclear cells

Peripheral blood was obtained from patients and normal healthy laboratory volunteers using a 19 gauge needle. Mononuclear cells were then isolated from the fresh, heparinized peripheral blood. Blood was diluted with an equal volume of PBS and layered onto one third the volume of Ficoll-Isopaque (density = 1.086 gcm^3) and centrifuged for 30 minutes at 400xg. Following centrifugation, the upper phase was carefully removed and discarded. The interface was collected and subsequently washed three times in magnesium free Hanks balanced salt solution (HBSS) (Gibco). Adherent cells were removed by culture of the washed interface in 75cm^2 tissue culture flasks for 1-2 hr. at 37^{0}C. The non-adherent cells were recovered, counted and re-suspended at a density of 1x10^{6}ml^{-1} in complete medium. Cells not required for immediate use were frozen in liquid nitrogen as described below.

2.4 Cell freezing, storage and thawing

Cells obtained from peripheral blood, fresh tumour samples, bladder cancer cell lines and other cell lines were stored in a liquid nitrogen filled vivo-stat. Washed cells were re-suspended at $2x10^6ml^{-1}$ in 90% foetal calf serum containing 10% dimethlysulphoxide (DMSO). Aliquots of 1ml were placed in freezing vials (Gibco) and these placed in polystyrene boxes. The boxes were transferred to a -70°C ultra low temperature freezer overnight prior to long term storage in liquid nitrogen until required (-196°C).

Cells were recovered from long term storage by rapid thawing to 37°C in a water bath. The 1ml aliquot was then washed twice with complete medium in order to remove any cell debris and DMSO. Using dye exclusion the viability of each vial was assessed prior to culture. Vials with a cell viability of less than 95% were discarded.

2.5 Cytokines

The cytokines used during these studies were of the highest obtainable quality. All cytokines were human recombinant *E. coli.* derived and of high purity containing less than 2 endotoxin units per mg of cytokine protein, unless otherwise stated. The details of their specific activity, source and unit definition are as follows;

Interleukin-1 alpha (IL-1α);

Recombinant IL-1 was obtained from British Biotechnology Ltd. and contained $5x10^8$ Umg-1 protein. One unit was defined as the quantity of IL-1 required to produce 50% maximal tritiated thymidine incorporation in the mouse helper T-cell line D10.G4.1.

Interleukin-2 (IL-2);

Recombinant des-alanyl-1, serine-125 IL-2 was generously provided by Euro-Cetus B.V. and contained $1.8x10^7 Umg^{-1}$ protein designed for clinical administration. One unit was defined as the amount of IL-2 required to produce half-maximal tritiated-thymidine incorporation in cultured HT-2 cells.

Interferon-alpha 2a (IFNα2a);

Recombinant IFNα was the generous gift of Dr. S. Langden, ICRF, Edinburgh, and contained $2x10^7 Umg^{-1}$ protein. One unit was defined as the quantity required to produce equivalent anti-viral activity to that expressed by 1 unit of NIH reference standard.

Interferon-gamma (IFNγ);

Recombinant human IFNγ was purchased from Boehringer Mannheim and contained $2.5x10^7 Umg^{-1}$ protein. One unit was defined as the amount of IFNγ required to produce equivalent anti-viral activity to 1 unit of NIH reference standard Gg 23-901-530.

Tumour Necrosis Factor-alpha (TNFα);

Recombinant human TNFα was the kind gift of Behringwerke and contained $5.1x10^{10} Umg^{-1}$ protein. One unit was defined by the half-maximal cytotoxicity against the murine fibroblast cell line L929 in the presence of actinomycin-D.

Transforming Growth factor-beta2 (TGFβ2);

Recombinant TGFβ was purchased from Genzyme Corp and contained Umg^{-1} protein. One unit was defined as the quantity of TGFβ

required to produce 50% maximal tritiated thymidine incorporation into the mink lung epithelial cell line Mv1Lu

2.6 Generation of LAK cells

Non-adherent peripheral blood mononuclear cells (PBMC) were cultured at a density of $1x10^6ml^{-1}$ in complete medium. LAK cells were generated by the addition of exogenous recombinant IL-2 (and other cytokines) at concentrations up to $1,000Uml^{-1}$. The mononuclear cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for up to 6 days. After the incubation the cells were washed twice in HBSS and resuspended at $4x10^6ml^{-1}$ and assessed for cytolytic activity against labelled target cells.

2.7 Cytotoxicity Assay

Cytotoxicity was measured by means of a 4 hour chromium-release assay. In brief, 10^6 target cells were radiolabelled with 100 μCi $Na^{125}ICrO_4$ (Amersham International) at 37°C for 1-hr with shaking. After three washes in HBSS the targets were resuspended in RPMI 1640 at a density of $5x10^4ml^{-1}$. Labelled targets ($5x10^3well^{-1}$) were dispensed into the wells of 96-well U-bottomed microtitre plates (Sterilin Ltd) and then graded numbers of effector cells were added to give effector-to-target ratios (E:T) ranging from 40:1 to 1.25:1 in a total of 200μl of medium. The spontaneous release of chromium from labelled target cells was determined by culture in the absence of effector cells for the duration of the

assay. Maximal release was determined by the addition of 1M sodium hydroxide to labelled targets. After a 4 hr. incubation at 37°C and centrifugation at 100xg for 5 minutes, 100 µl of supernatant was removed from each well and the radioactive content determined using a liquid scintillation counter (Canberra Packard Ltd). All assays were performed in triplicate and the data were calculated from E:T of 40:1 in order to allow comparison between lines of differing susceptibility. Results were expressed as the percentage specific cytotoxicity, calculated as below;

$$\%\text{Specific Cytotoxicity} = \frac{\text{cpm exp release - cpm spontaneous release}}{\text{cpm total release - cpm spontaneous release}}$$

The natural killer (NK) cell sensitive target K562, an erythroleukaemic cell line, was used as the positive control for NK activity resident in PBMC. The B lymphoblastoid cell line, Daudi, which is insensitive to NK activity was used as the positive control for LAK activity.

2.8 Preparation of mononuclear cells from human spleen and lymph nodes

The spleen and mesenteric lymph nodes were removed from cadavers who had been selected as multi-organ donors. Tissue was placed in sterile PBS for transfer to the laboratory. Connective tissue was first removed from the organ following which slices of tissue were placed in a petri dish and surplus blood removed by washing with PBS. Using a pair of 19 gauge needles the tissue was gently teased apart and minced until a

fine unicellular suspension was obtained. Large pieces of tissue were discarded and the suspension of cells layered onto a ficoll density gradient as described for the preparation of PBMC. Cells harvested from the interface were washed and stored frozen until required.

2.9 Source of Monoclonal Antibodies

For details of antibody specificity and source see table 3. For flow cytometry, monoclonal antibodies which were not directly conjugated to a fluorochrome were detected using a goat anti-mouse-IgG $F(ab')_2$ fragment fluorescein isothiocyanate (FITC) conjugate (Sigma Chemical Co).

2.10 Fluorescence Staining & Flow Cytometric Analysis

Single and dual colour immunofluorescence was performed on cells stained indirectly or directly with monoclonal antibodies conjugated to either fluorescein isothiocyanate (FITC) or PE (phycoerythrin). For each analysis $1x10^5$ cells were washed with washing buffer (PBS, 1% FCS, 0.01% sodium azide) and incubated at 4°C for 30 minutes with optimal concentrations of primary monoclonal antibody. Following two washes by centrifugation, the binding of unconjugated monoclonal antibodies was detected using a sheep anti-mouse-FITC conjugate for a further 30 minutes at 4°C. Cells to be stained for two colour analysis were then blocked with a 10% (v/v) solution of normal mouse serum for 30 minutes and, after washing, incubated with a second monoclonal antibody conjugated to PE for a further 30 minutes. The cells were washed twice

Table 3. Details of the antibodies used in these studies

Antigen	Alternative name	Source	Dilution for flow cytometry
CD2	-	I	1:50
CD3	-	I	1:50
CD4	-	I	1:50
CD8	-	I	1:50
CD11a	LFA-1α	II	1:50
CD16	FcγIIIR	III	1:100
CD18	LFA-1β	II	1:50
CD22	-	I	-
CD25	IL-2R	III	1:100
CD54	ICAM-1	IV	1:200
CD56	NCAM	II	1:50
ICAM-2	-	IV	1:100
EGF-R	-	V	1:400
MHC class I	-	II	1:50
MHC class II	-	VI	1:64
VCAM	-	VII	1:50
IFNγ*	-	VIII	-
TNFα*	-	VIII	-

The table provides details of the monoclonal and polyclonal (*) antibodies used in the studies. The name of the antigen and alternative name (if any) is given. The sources of the antibodies are as follows; I - Scottish antibody production unit, II - Dako, III - Becton Dickenson, IV - Dr. T. A. Springer, V - Genzyme, VI - Dr. I. Trowbridge, VII - British Biotechnology, VIII - Dr. A. Meager (NIBSC). Also shown is the dilution of monoclonal antibody which was used for flow cytometric staining procedures.

more and resuspended in 1% para-formaldehyde prior to analysis using a flow micro-fluorometer (Coulter). Non-viable cells were gated out of the window and at least 10,000 events were accumulated by using logarithmic amplification of fluorescence intensity.

2.11 Isolation of fresh tumour cells

Viable tumour cells were obtained from bulky fragments selected from cytoscopically resected bladder tumours. Debris and blood clots were first removed and the tumour was washed with PBS. Soft, friable, papillary tumours were gently teased apart using tweezers and scissors until a unicellular suspension was formed. The larger fragments were allowed to settle-out and the single cell suspension was layered onto ficoll in the manner described for the preparation of PBMC. Cells at the interface were collected and washed and stored frozen until required. Cytospins of these cells were examined by a consultant pathologist and deemed to consist almost entirely of tumour cells.

2.12 Dyes

Target cells were labelled with the dye hydroethidine (HE) (Molecular Probes). After penetration of the cell HE is enzymatically oxidised and intercalated into DNA as a red dye. The maximum emission wavelength of intercalated ethidium is 610 nm. HE was prepared as a stock solution of 253mM in N, N-dimethyl acetamide and stored at 4°C protected from the light. On the day of assay, a working solution was prepared by diluting the stock solution 1:1000 in PBS. Since this concentration was close to saturation, the solution was filtered through a

0.22μm membrane to remove precipitates.

Effector cells were labelled with the stain carboxyfluorescein diacetate acetoxymethylester (CFDA.AM) also from Molecular Probes. CFDA. AM is a vital stain and a pH probe (pKa=6.4) which stains the cell cytoplasm green. Esterified CFDA.AM produces a fast cell staining which is enzymatically modified within the cell by esterases. The maximum emission wavelength for CFDA is 520nm. The stock solution was 2.5mM in DMSO and was stored at 4°C in the dark. Working solution was prepared by diluting 1:40,000 in PBS. Both dyes can be excited at a common wavelength (488nm) using a single argon laser.

2.13 Staining procedure

Target and effector cells were washed and resuspended at $2x10^6$ cells ml^{-1} in the working solution of the appropriate dye (HE: 253μM, CFDA.AM: 62.5nM). The cells were incubated for 30 minutes at 37°C with agitation every 10 minutes. Staining was stopped by washing twice in PBS. The final wash was performed in the assay buffer of choice and cell concentrations were adjusted to the yield appropriate effector to target ratios. Labelled cells were stored at 20°C protected from the light.

2.14 Quantification of effector/target conjugate formation

Conjugates were formed by mixing 100μl each of effector and target cells in a 0.5ml eppendorf tube, pelleting rapidly by centrifugation and vigorously resuspending using a vortex machine. The ratio of effector to target cells was maintained at 10:1. The formation of conjugates was assessed immediately after pelleting (t=1) and at 5 minute intervals

thereafter by flow cytometry. Control for background conjugation was performed by mixing effector and target cells without pelletting (t=0). In all controls the number of background conjugates recorded was low, usually <2% of the experimental conjugates. Further controls involved the analysis of effector or target cells alone. Finally, the extent of homophyllic conjugation was determined by assaying effector or target cells, part of each cell type being stained with HE and the other part with CFDA.AM.

2.15 Flow cytometric analysis of conjugate formation

Flow cytometric analysis was performed using an EPICS-C flow cytometer (Coulter) operating at 488nm and 200mW output. A 70μm aperture was used with a low differential pressure between sheath and sample. Three different parameters were studied; namely forward angle light scatter, red and green fluorescence. The forward angle light scatter was used to trigger analysis and to gate all other signals from debris smaller than lymphocytes. Controls with stained target and effector cells were performed separately to check the settings of the photomultiplier tube gains.

Analysis was performed and halted when 1,000 target events (red) were recorded. The quantification of conjugate formation based on a fixed number of target cells at a standard effector to target cell ratio was a fundamental element of the procedure.

2.16 Antibody blocking studies

Labelled effector and/or target cells were pre-incubated with monoclonal antibodies ((5μgml^{-1}) at 20°C for 30 minutes) to various

adhesion molecules prior to the formation of conjugates by centrifugation. Control reactions were performed with an irrelevant monoclonal antibody (anti-CD22).

2.17 The role of divalent cations and energy dependent processes

The role of various divalent cations in the formation of conjugates was assessed using calcium, magnesium, and manganese chloride dissolved in 0.15M NaCl. EGTA and EDTA (both 1mM in 0.15M NaCl) were used to chelate calcium ions and other divalent cations from the assay buffer of choice. Immediately following the addition of either EGTA or EDTA to the conjugation buffer, conjugates were formed by centrifugation. The medium removed and replaced by PBS containing 1%FCS.

In addition to cation chelation, varying concentrations of divalent cation chloride solutions were added to the conjugation buffer prior to centrifugation to give final concentrations ranging between 0.1 and 16 mM. Sodium azide was also briefly added to the conjugation buffer at a concentration of 0.1%. Following centrifugation, the cells were resuspended in PBS containing 1% FCS.

2.18 Source of cytokines used in receptor studies

Highly purified recombinant mono-iodinated IFNγ was purchased from Amersham International and had a specific activity of approximately 760Ci $mmol^{-1}$. The precise specific activity of each batch of radio-ligand was determined prior to each assay. The percentage of free iodine in each batch of radio ligand was determined by thin layer chromatography. The

percentage free iodine was never greater than 2.5%. Radioligand was discarded if not used within four weeks of the date of manufacture.

2.18 Determination of optimal number of cells

The number of cells in each assay was fundamental to the sensitivity and accuracy of the subsequent Scatchard analysis. Cell monolayers were dispersed using 0.02% EDTA for 15 minutes. For each cell line to be examined, varying numbers of cells ranging from 10^4-10^6 were placed in plastic RT30 tubes in triplicate. To each triplicate 0.3ng radioligand was added and allowed to equilibrate for 4 hours at 4°C. Following incubation the cells were washed with washing buffer (HBSS, 0.1% BSA (Sigma)) and the pellet associated radioactivity measured on a multi-gamma counter (LKB).

2.19 Determination of optimal number of washes and optimal time

The optimal number of washes was investigated in order to maximize the difference between the non specific binding and the specific binding. Triplicate tubes containing $2x10^5$ cells were incubated for varying durations with 0.3ng of radioligand in the presence and absence of a 100 fold excess of unlabelled ligand. Following incubation at 4°C, the cells were washed repeatedly with washing buffer. The radioactive content of each tube was determined following each wash.

2.20 Positive and negative controls for binding studies

In order to confirm the specificity of the assay technique used,

positive controls in the form of human Daudi cells which had previously been assessed for IFNγ receptor expression were used. The negative control cells were normal human erythrocytes which were presumed not to bear the IFNγ receptor.

2.21 Competitive binding experiments

Competitive binding experiments were carried out in plastic RT30 tubes. Cell monolayers were washed with PBS before incubation with 0.02% EDTA for 15 minutes. Trypsin was not used as damage to receptor structures may have occurred. Released cells were washed and resuspended at $4x10^5ml^{-1}$ and incubated for the indicated times in binding medium (complete medium) in the presence of a fixed concentration of radiolabelled IFNγ and increasing amounts of unlabelled IFNγ for 2 hours at 4°C. The total reaction volume was 500µl and the concentration of radioligand was 0.3ng/ml. Non-specific binding was determined in the presence of a 100 fold excess of unlabelled ligand. Binding was terminated by washing the cells twice with ice-cold washing buffer. Cell bound radioactivity was determined using a gamma-counter. Each experiment was performed in triplicate.

The binding data were analyzed and calculated manually. Binding curves and subsequent Scatchard plots were drawn using Cricket Graph software. The correlation coefficients of Scatchard plots were always significant to between the $p<0.01$ and $p<0.001$ level. Using a linear regression of the Scatchard plot, analysis of the number of binding sites per cell (B_{max}) and the dissociation constant (K_d) was carried out assuming that the molecular moieties of IFNγ which bound to the cells were dimers of molecular weight 34,400 Da, thereby giving a minimal estimate of the

receptor density. If the monomeric form was to bind, the number of binding sites per cell would simply be doubled. All experiments were carried out a minimum of 6 times and the results expressed represent the mean ± standard deviation.

2.22 Determination of surface associated and internalized ligand

To monitor the rate of internalization of IFNγ, $2x10^5$ cells were incubated, either in suspension or as adherent monolayers, with approximately 0.3ng of ^{125}I-labelled ligand at 37°C. Binding was terminated by washing the cells twice at 4°C with washing buffer. Cells were then incubated for 4 minutes at 4°C with 0.5M NaCl (titrated to pH 2.5 with acetic acid) in order to "strip" the membrane associated (acid-dissociable) ligand. The cells were then centrifuged and the supernatant and pellet associated radioactivity determined separately. The radioactive content of the pellet was assumed to be due to internalized ligand.

2.23 Reagents for experiments concerning signal transduction

Fura-2AM, PMA, calcium ionophore A23187, ionomycin, H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine), W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide), Diltiazem, TMB-8 (8-(diethylamino)-octyl 3, 4, 5-trimethoxybenzoate hydrochloride), Digitonin, EGTA, and staurosporine were purchased from Sigma Chemical Co. and were of the highest obtainable grade.

2.24 Induction of MHC class II and ICAM-1 molecules

Bladder cancer cells were cultured in 24-well, flat bottomed culture plates (Costar) at $2x10^5$/well at 37°C in a humidified atmosphere of 5% CO_2 in air. IFNγ was used at a concentration of $500Uml^{-1}$ for 48 hrs and $100Uml^{-1}$ for 24 hrs in the case of MHC class II and ICAM-1 induction respectively. After incubation, cells were washed twice with ice cold PBS and harvested for analysis of expression of HLA class II and ICAM-1 molecules on their surface.

2.25 Depletion of extracellular calcium

Cells were cultured in complete medium containing 1mM EGTA for 1 hr before the addition of IFNγ. As a control, $CaCl_2$ at 1.424 mM was added into the EGTA-containing medium to recover the amount of free calcium to the original concentration in RPMI 1640.

2.26 Measurement of free cytosolic calcium flux

The cytosolic free calcium concentration of human TCC cells was monitored using the calcium indicator Fura-2AM. The cell lines ($1x10^6$/ml) were loaded with 5μM Fura-2AM for 20 minutes at 37°C. Following loading the cells were washed twice in HBSS before resuspension in HBSS. TCC cells ($1x10^6$/ml) were placed in temperature controlled cuvettes with continuous magnetic stirring. Cells were stimulated with interferon-gamma and/or various pharmacological agents for 30 minutes at 37°C. Fluorescence was monitored on a Perkin-Elmer LS-50 spectrofluorometer with the excitation and emission wavelengths set at 340 nm and 510 nm respectively. The calcium concentration was determined using a dedicated computer where the K_d for the calcium-Fura-2 complex at 37°C is 225 nm.

The maximum fluorescence was obtained by disruption of the cells with digitonin and the minimum fluorescence obtained by the subsequent addition of 5 mM EGTA, pH 8.0 (final concentration).

2.27 Detection of soluble ICAM-1 in tissue culture supernatants

The presence of soluble ICAM-1 in tissue culture supernatants was determined using a sandwich ELISA test kit (T-cell Diagnostics). Briefly, samples of cell-free tissue culture supernatant were incubated in the wells of a microtitre strip plate for 2 hours. Following washing, the presence of soluble ICAM-1 was detected using a second monoclonal antibody to ICAM-1, conjugated to HRP.

2.28 Assessment of the rate of proliferation

The growth rate of tumour cell lines in response to various stimuli was determined by monitoring the incorporation of tritiated (^3H) thymidine into newly synthesized DNA. Cells were seeded and grown in 96-well, flat-bottomed culture plates until they reached the log phase of growth (48 hrs.). Complete medium was then replaced with medium supplemented with appropriate concentrations of various cytokines. Subsequently, at various time points up to 100 hours wells were harvested in sextuplets. Prior to each harvest, the wells were pulsed for 12 hours with 0.5μCi of H3-thymidine (Amersham). Cells were harvested after trypsinization using an automated cell harvester (Skatron) onto glass fibre filter discs. After drying, the radioactive content of each disc was determined using a liquid scintillation counter (Tri-Carb 1900CA, Canberra-Packard).

2.29 Patient details

Six patients with superficial bladder cancer were treated using six weekly instillations of intravesical Evans BCG vaccine (formerly Glaxo strain). Immediately before BCG instillation the bladder was voided. Each instillation consisted of $1\text{-}5x10^9$ colony forming units of BCG suspended in 60 ml of 0.9% (w/v) saline. The solution is retained for 2 hours in the bladder. Routine cold-cup biopsy were taken for histological examination before the course began and six weeks after the final instillation.

2.30 Preparation and storage of patients urine

Urine from the twelve hours following each BCG instillation was collected and processed within 12 hours. The volume of urine was measured and the pH determined. Cellular debris was removed by centrifugation at 2,000 rpm for 10 minutes. The cleared sample was then dialysed through a 10kDa membrane against several changes of phosphate buffered saline (pH 7.2) overnight at 4°C. The pH of the sample was determined and the sample was finally filtered through a 0.22μm syringe filter and stored aliquoted at -70°C.

2.31 Preparation of frozen sections of bladder tumour

Biopsy material and pieces of tissue obtained after cystoscopic resection of tumour were collected and snap frozen in liquid nitrogen. Five μm frozen sections were cut at -25°C, air dried overnight and fixed in fresh 100% acetone at room temperature for 2 hours. After further air drying

they were wrapped in aluminium foil and stored desiccated at -20°C.

2.32 Immunohistochemical method

Frozen sections were recovered from -20°C storage and rehydrated with 0.2M tris buffered saline (TBS) pH 7.6 for 10 minutes. Following rehydration the slide around the section was wiped dry and 100µl of an optimal dilution of primary monoclonal antibody was applied over the section. The slides were incubated at 20°C in a humidified atmosphere for 60 minutes following which they were carefully washed twice in TBS. Binding of the primary monoclonal antibody was detected using a 1:50 dilution of rabbit anti-mouse immunoglobulin pre-conjugated to horse radish peroxidase (HRP) (Dakopatts) for 30 minutes. This antibody was prepared in 10% normal human serum and pre-incubated for 30 minutes at 20°C. After washing twice with TBS, the slides were flooded with substrate solution (diaminobenzidine DAB (3mg in 5ml DAB buffer, pH 7.6) (Sigma Chemical Co.) which had been activated with 50µl of 3% hydrogen peroxide solution in distilled water. After approximately 10 minutes the substrate was removed by washing with TBS and the sections were lightly counter stained using Mayer's Haematoxylin (BDH Chemical Co.) for 2 minutes. The counterstain was "blued" by washing in alkaline tap water (pH 8.0) until all the excess stain had been removed. The sections were then progressively dehydrated by immersing in 30%, 70% and 100% ethanol solutions for 1.5 minutes each prior to washing in sulphur free xylene. The sections were then wet mounted using DPX mounting medium (BDH).

2.32 Sample preparation for electron micrography

Cells were grown on plastic slides for 48 hours following which LAK cells were plated on top and incubated for 30 minutes in a tissue culture incubator. After incubation the slides were washed with PBS (pH7.2) and stored in glutaraldehyde. Fixed slides were freeze dried and splutter coated with gold particles. Samples were scanned and photographed on a Phillips 505 electronmicroscope (Phillips).

2.34 L929 Bioassay for tumour necrosis factor

The murine L929 fibroblast cell line, which is exquisitely sensitive to the cytotoxic effects of TNF was used to determine the biological activity of TNF in urine samples. Briefly, L929 cells were seeded in 96 well plates and cultured for 48 hours. Standards and samples were then added and serially diluted across the plate. After a further 48 hours, 10µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide ($1mgml^{-1}$) was added to each well and incubated for 2 hours at 37°C. The supernatant was removed and replaced with 50µl of acidified isopropanol to dissolve the blue crystals. After complete dissolving had occurred the optical density was determined at 490nm and a standard curve constructed from the results. Test results were interpolated with the standard curve.

3 Results

3.1 The sensitivity of bladder cancer cells to natural and cytokine enhanced cellular cytotoxicity

Although previous workers have demonstrated the ability of LAK cells to lyse a variety of human tumour targets, little attention has been paid to urological malignancies, with the exception of renal cell carcinoma. It was our intention to study the potential killing of bladder cancer cells by LAK cells and to investigate the role of cytokines in this activity.

3.1.1 Bladder cancer cells are not susceptible to NK mediated lysis

Natural killer cells can mediate the cytolysis of a limited number of tumour targets *in vitro*. When established bladder cancer cell lines were co-incubated with freshly isolated non-adherent PBMC in a standard chromium release assay, no specific cytotoxicity was observed within the 4 hour assay period. However the NK sensitive K562 cell line was lysed. At a 40:1 effector to target ratio the observed specific cytotoxicity was 60%±10. On the other hand, the NK insensitive Daudi cell line was not killed by fresh PBMC.

3.1.2 The dose response and kinetics of LAK cell induction by IL-2

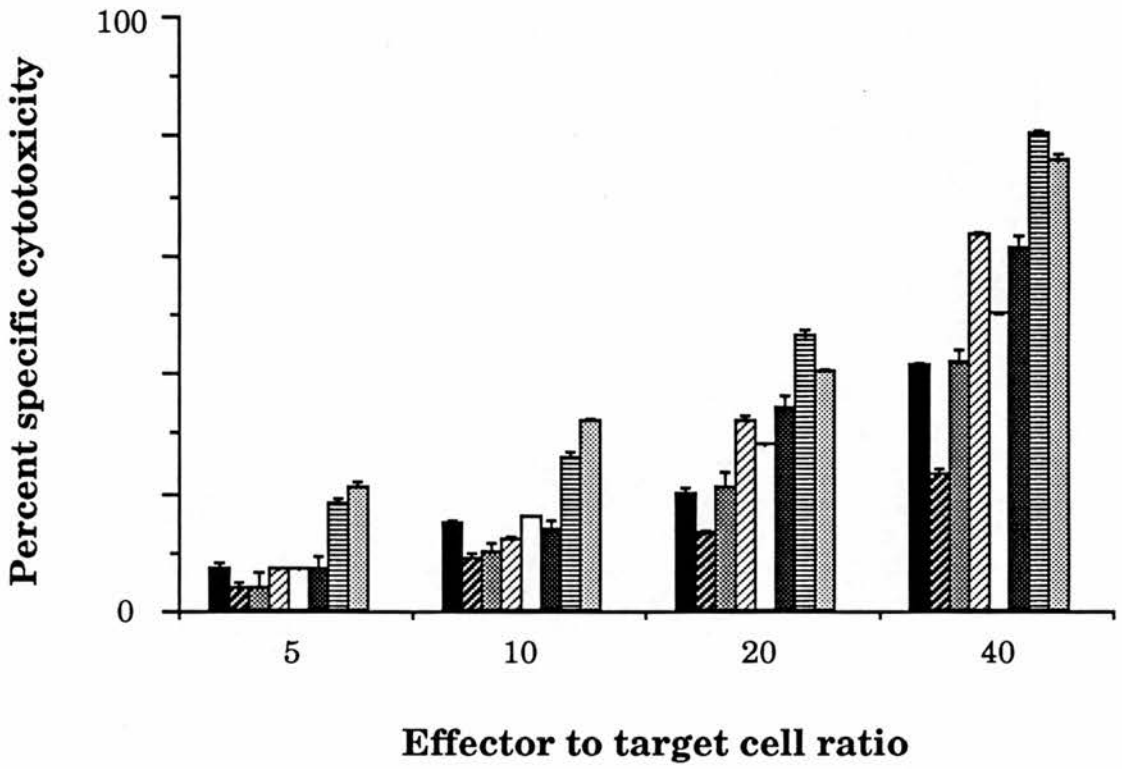
During the past decade many investigators have examined the ability of IL-2 activated LAK cells to mediate the cytolysis of a wide variety of tumour cell *in vitro*. Central to this project is the susceptibility of bladder cancer cells (both freshly derived and established cell lines) to

LAK mediated cytotoxicity. Therefore, the ability of IL-2 activated killer cells to lyse bladder cancer targets was examined.

Firstly, the influence of varying the ratio of effector to target cell was investigated. When the effector to target ratio was low (5:1) the was little detectable cytotoxicity against the majority of the cell lines (Figure 7). However, increasing the effector to target ratio showed the susceptibility of the different cell lines. At a ratio of 40:1 the differential susceptibility of the target cells was most evident, therefore, it was decided that this ratio was most suitable for further experiments. As mentioned, the target cells were not equally sensitive to LAK mediated cytolysis. Table 4 shows the maximal achievable specific cytotoxicity against the eight cell lines. The least susceptible cell line was RT112, with J82 and UMUC3 being several times more sensitive to the same effector cells. The histopathological grade of each cell line is also shown in table 4. There was no apparent relationship between the susceptibility of each line to LAK mediated killing and the grade of the parent tumour.

Depending on the workers involved and upon the source of recombinant IL-2 used, the concentration of IL-2 required in order to generate maximal LAK activity ranges between 1 and 1,000Uml^{-1}. The effect of increasing concentration of IL-2, and the duration of activation on LAK activity in our studies is shown in Figure 8. After 3 days of culture in the absence of IL-2, a significant increase in LAK activity above day 0 was evident. Dose dependent LAK activity was also observed following 3 days of continuous activation with IL-2. This activity was maximal when cells were cultured with 1,000Uml^{-1} IL-2. Increased LAK activity was seen following 6 days of activation. Any further increase in either the concentration of IL-2 or the duration of activation failed to result in a corresponding increase in lytic potential (data not shown).

Figure 7. The effect of effector to target ratio on LAK activity



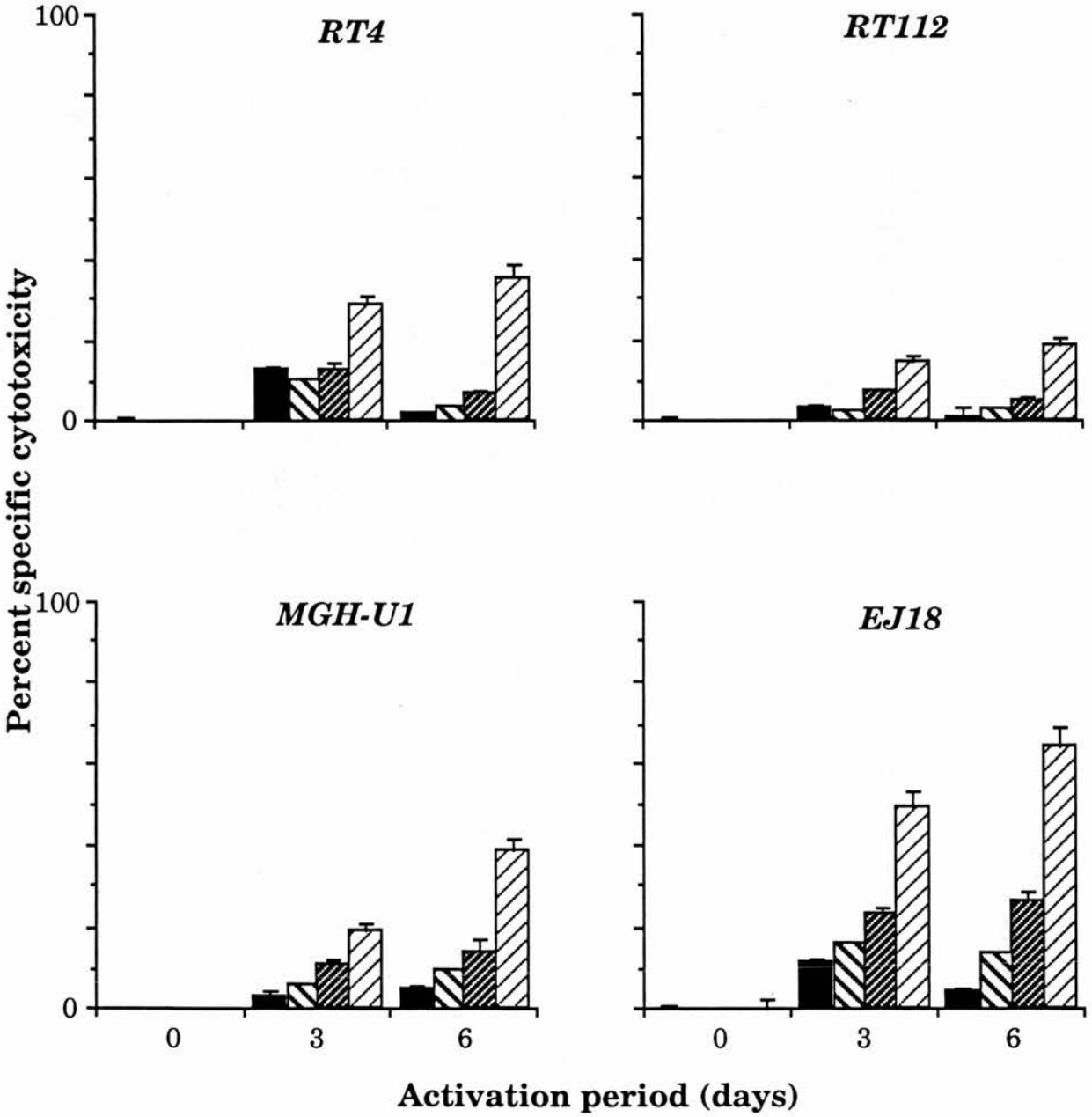
The maximal achievable specific cytotoxicity against a panel of eight bladder cancer cell lines at various effector to target cell ratios. Effector cells were generated by incubation of PBMC with 1000U/ml IL-2 for 6 days, these cells were then tested for their ability to lyse bladder cancer cell lines in a standard 4 hour chromium release assay. Bars represent the mean of at least triplicate determinations, error bars show standard error. The cell lines tested were; RT4 (■), RT112 (▨), MGH-U1 (▩), EJ18 (▨), SD (□), 5637 (■), J82 (▤), and UMUC-3 (▩).

Table 4. The susceptibility of bladder cancer cell lines to LAK mediated cytolysis

Cell line	RT4	UMUC3	RT112	MGHU1	EJ18	J82	SD	5637
Grade	G1	G1	G2	G3	G3	G3	G3	G3
Specific Cytotoxicity	40	76	24	40	65	80	50	61

Shown are the mean results obtained with the standard cytotoxicity assay for the percent specific cytolysis against eight bladder cancer cell lines. The results are those obtained with an effector to target ratio of 40:1 and represent the mean of at least five determinations in triplicate. For the cell lines RT4, RT112, MGH-U1 and EJ18 the number of determinations exceeded 20.

Figure 8. The dose dependence of LAK induction by IL-2



The dose dependent kinetics of LAK activity induction by IL-2. PBMC were incubated for the indicated periods with increasing concentrations of IL-2; 0U/ml (■), 10U/ml (▧), 100U/ml (▨), and 1000U/ml (▨). LAK activity was then determined against four bladder cancer cell lines using a standard 4 hour chromium release assay. Bars represent the mean of triplicate determinations and error bars represent the standard error of the mean.

3.1.3 The variability of different donors for LAK cells

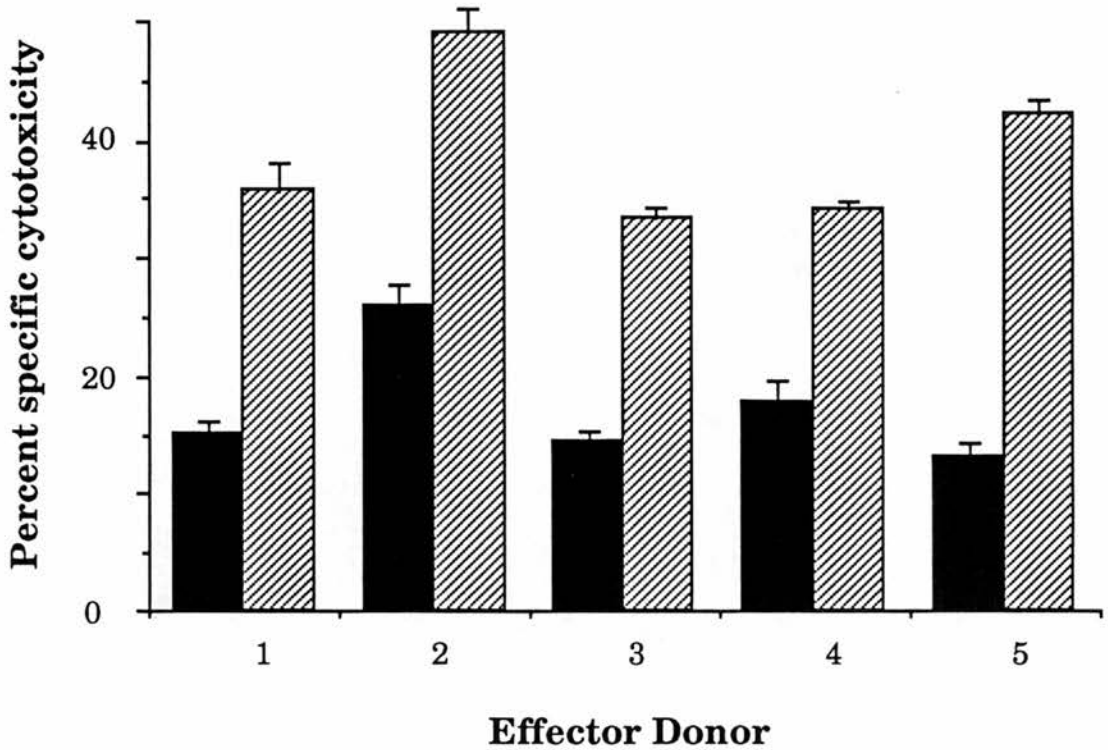
The cytotoxic potential of LAK cell derived from different donors was simultaneously assessed against two TCC cell lines (Figure 9). Differences in the maximum achievable specific cytotoxicity were evident between cells from different donors. The mean level of specific cytotoxicity against RT112 (G2) cells was 18.2%±4, and against MGH-U1 (G3) cells 36.6%±3.9. One donor, number two, had levels of cytotoxicity which were greater than those achieved by cells from the other donors, however, the difference in susceptibility between the two lines was maintained.

3.1.4 The effect of differing lymphoid organ sources for LAK cells

The effect of lymphoid source for LAK cell precursors was investigated by using the spleen and lymph node obtained from cadavers where consent for multi-organ donation had been given. Following activation for six days with IL-2 there was no discernible difference between LAK cells generated from the spleen or the lymph node (Figure 10).

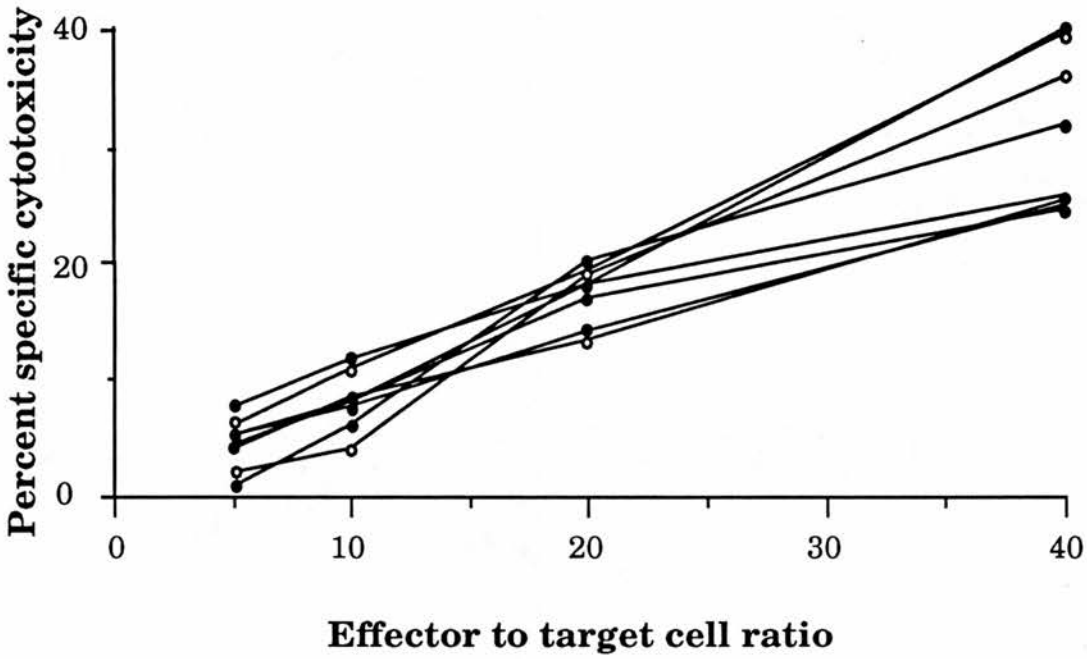
Unfortunately, considerable difficulty was encountered in obtaining lymphocytes from the peripheral blood sample and therefore no data for this source of cells exists. However, the results obtained with spleen and lymph node are not dissimilar to those obtained with LAK cells derived from the peripheral blood of healthy volunteers. As with the different donors of PBMC (see above), small differences in the achieved specific cytotoxicity were evident between the donors.

Figure 9. The effect of different donors for LAK cells



The simultaneous assessment of the cytotoxic ability of LAK cells derived from five different donors against two different target cell lines; RT112 (■), MGH-U1 (▨). LAK cells were generated by activating non-adherent PBMC with 1000U/ml of recombinant IL-2 for 6 days. LAK activity was assessed using a standard 4 hr. chromium release assay. Shown are the mean of triplicates with error bars indicating 1 standard deviation.

Figure 10. The influence of different lymphoid organs as source of LAK cells



The effect of different lymphoid organs as the source tissue for LAK cell precursors. The spleen (open symbol) or lymph node (closed symbol) from four cadaver were collected and processed to yield a unicellular suspension. This was activated with IL-2 for six days and the resulting LAK activity determined against the bladder cancer cell line RT112.

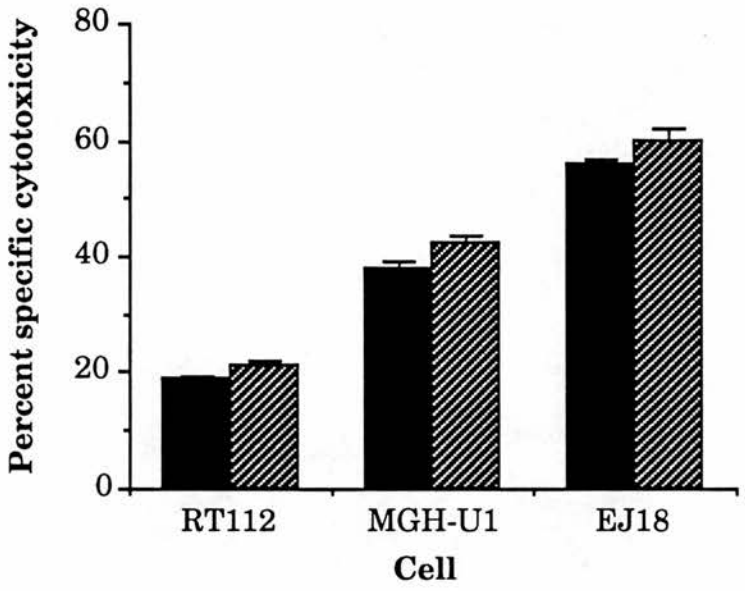
3.1.5 The effect of foetal calf serum on LAK cell activity

Criticism has been levelled against workers who employed foetal calf serum in cytotoxicity assays and in the generation of LAK cells (personal communication, Lafreniere *et al* 1989). It was decided that this parameter should be briefly investigated before undertaking further studies. The presence or absence of foetal calf serum throughout the generation of LAK activity and during the assay did not have an effect upon the lysis of three cell lines (Figure 11).

3.1.6 Cytokines other than IL-2 can induce LAK activity

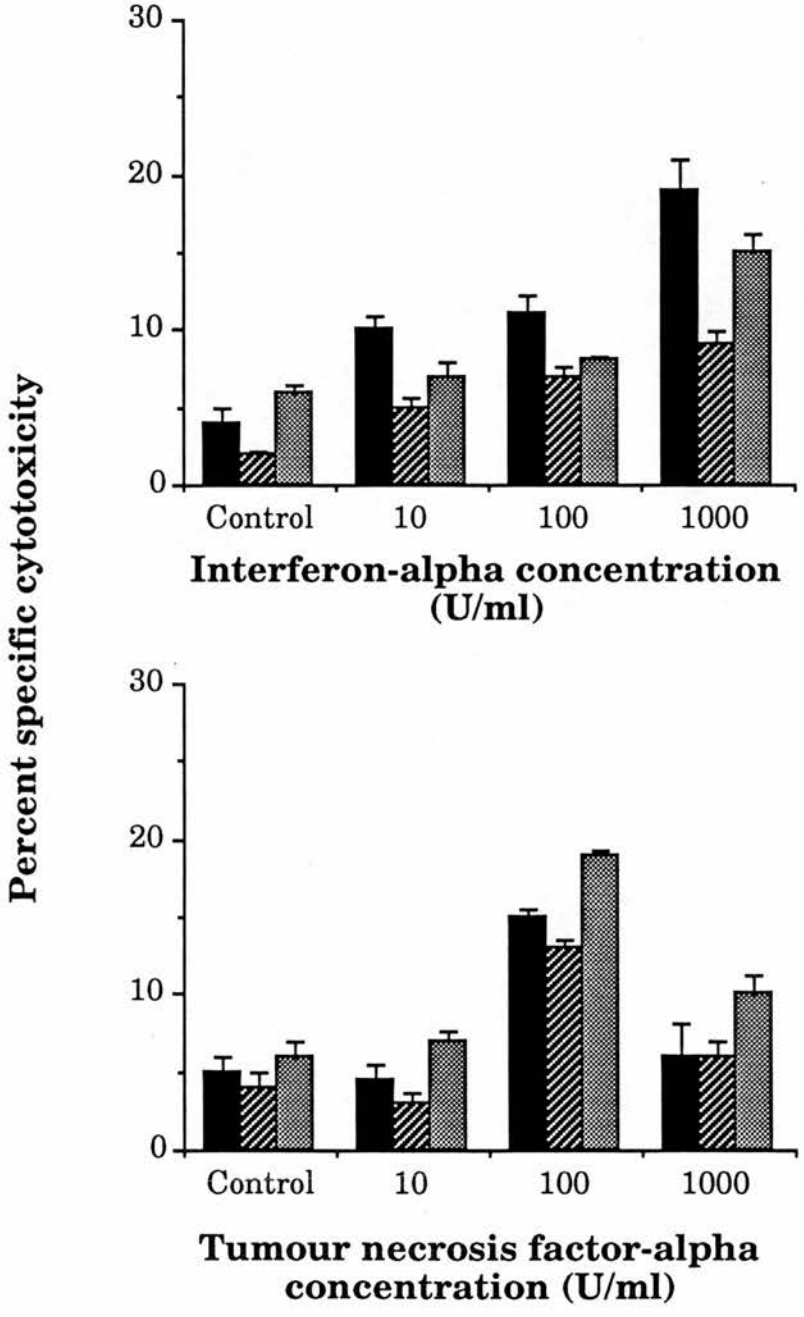
The initial reports concerning LAK activity showed that stimulation with IL-2 was required to generate such activity. However, other cytokines have been shown to be produced following stimulation with IL-2. The effect of these cytokines on LAK activity against three TCC cell lines was investigated. Both interferon-alpha-2a and tumour necrosis factor-alpha were found to stimulate LAK activity when used to stimulate PBMC for six days (Figure 12). The maximal levels of LAK activity against the three lines tested was, however, far less than that achieved with IL-2 (see above). The optimal concentration of IFNα was found to be 1,000Uml^{-1}, and for TNFα 100Uml^{-1}. When the concentration of TNFα was increased a further 10 fold, a decrease in specific cytotoxicity was observed.

Figure 11. The influence of foetal calf serum on LAK cell generation



The influence of foetal calf serum containing medium on the generation of LAK activity in PBMC following 6 days of activation in the presence of 1,000U/ml IL-2. LAK activity was determined against the indicated bladder cancer cell lines using a standard 4 hour chromium release assay. Bars represent the mean of triplicate determinations, error bars show 1 standard deviation. The presence of 5% FCS is shown (■), as is the effect of serum free medium (▨).

Figure 12. Cytokines other than IL-2 can induce LAK activity



The dose dependent kinetics of LAK cell induction by interferon-alpha and tumour necrosis factor-alpha. Freshly isolated PBMC were incubated for 6 days with the indicated concentrations of recombinant cytokine following which their LAK activity was determined against three bladder cancer cell lines; RT4 (■), RT112 (▨), and MGH-U1 (▩). Bars indicate the mean of triplicates and error bars indicate standard deviation.

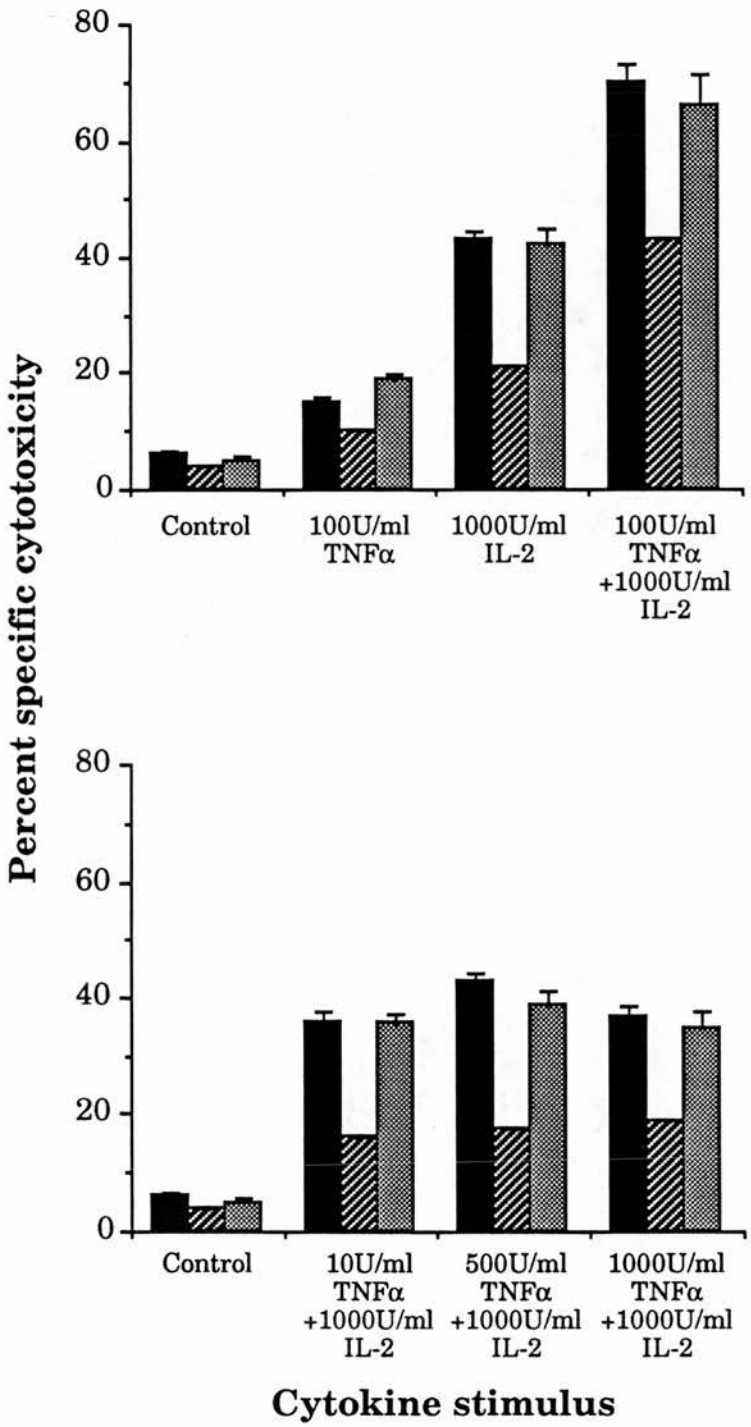
3.1.7 The role of exogenous tumour necrosis factor-alpha with IL-2 in the induction of LAK activity

From recent studies it is evident that cytokines other than IL-2 partake in the generation of LAK activity (Kaufmann *et al* 1991. Matossian-Rogers *et al* 1989). In order to investigate the possible role of multiple cytokines in the generation of LAK activity, the reported synergy between IL-2 and TNFα in LAK cell generation has been investigated. When LAK cells were generated by stimulation with both IFNγ and TNFα the resulting activity was greater than that achieved with either cytokine alone (Figure 13). However, this was only the case when TNFα was used at a concentration of $100Uml^{-1}$. Lower concentrations had no greater effect than that achieved with IL-2 alone, and higher concentrations resulted in a decrease from maximal LAK activity (Figure 13). There was no decrease in the viability of cells stimulated with the higher concentrations of TNFα as determined by dye exclusion (data not shown).

3.1.8 Interferon-alpha synergizes with IL-2 in LAK cell induction

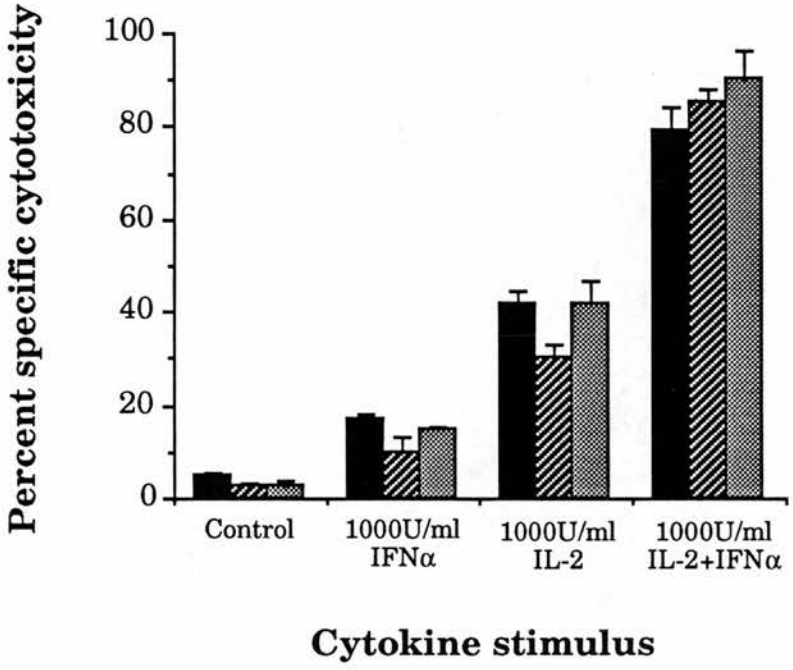
The number of reports on the role of IFNα in LAK activity are limited and there are no reports of synergy with IL-2 in the induction of such activity. When PBMC were stimulated with both IFNα and IL-2, at a concentration of $1,000Uml^{-1}$ each for six days, a greater LAK activity was observed against all three lines tested than with either molecule alone (Figure 14). The increase in activity was greater than the sum of that achieved by stimulation with either cytokine alone, thus indicating synergy in the induction of LAK activity. Concentrations of IFNα lower than $1,000Uml^{-1}$ were not observed to synergize with IL-2 (data not shown).

Figure 13. Tumour necrosis factor-alpha synergizes with IL-2 in the induction of LAK activity



The effect of IL-2 and TNFα on LAK activity against three bladder cancer cell lines , RT4 (■), RT112 (▨), and MGH-U1 (▩). LAK cells were cultured with the indicated concentrations of either IL-2, TNFα or a combination of both. LAK activity was determined in a standard chromium release assay. The upper figure shows the additive action of TNFα and IL-2, and the lower figure shows the effect of suboptimal concentrations of TNFα. Bars indicate the mean of triplicate determinations and error bars indicate the level of 1 standard deviation.

Figure 14. The synergy of IL-2 and IFNα in the generation of LAK activity



The synergy of IL-2 and IFNα in LAK activity against three bladder cancer cell lines , RT4 (■), RT112 (▨), and MGH-U1 (▩). LAK cells were cultured with the indicated concentrations of either IL-2, IFNα or a combination of both. LAK activity was determined in a standard chromium release assay. Bars indicate the mean of triplicate determinations and error bars indicate the level of 1 standard deviation.

3.1.9 LAK activity against bladder cancer in an autologous setting

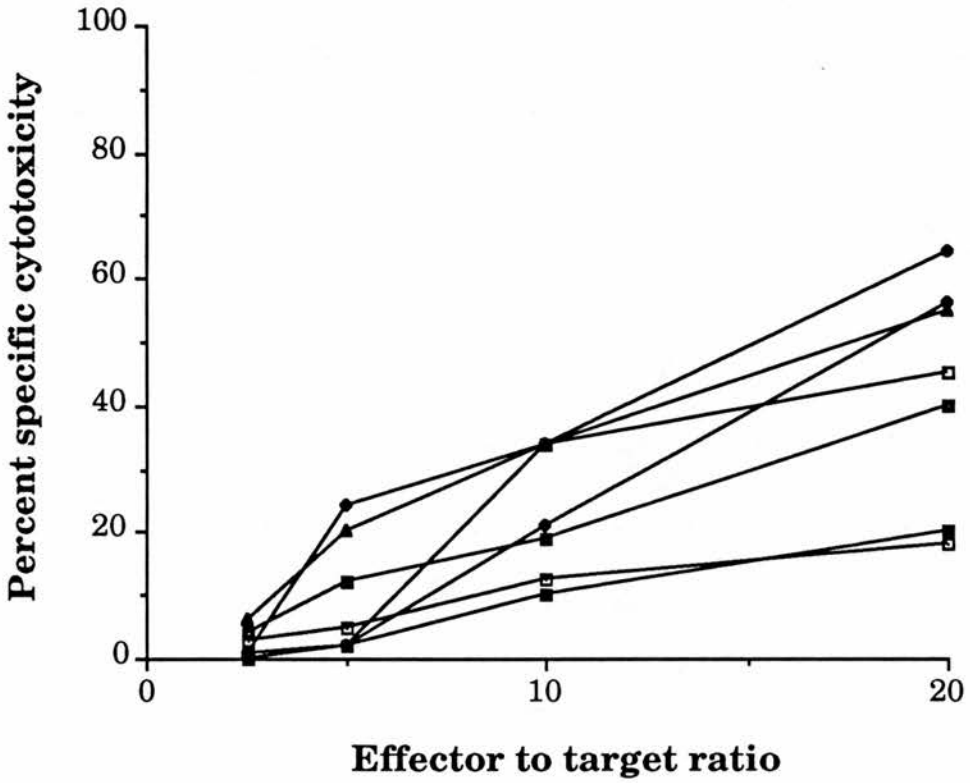
The cytolysis of bladder cancer cell lines is of limited usefulness if fresh bladder tumour cells are not susceptible to LAK activity. Therefore, the ability of autologous LAK cells to mediate the lysis of freshly derived tumour cells was investigated. The results obtained for the killing of tumour cells from seven patients by autologous LAK cells are shown in figure 15. The fresh tumour cells were differentially susceptible to LAK activity, as was found to be the case for cell lines. The maximal achievable specific cytotoxicity at an effector to target cell ratio of 20:1, was 62%±3. The mean level of achievable cytotoxicity was 42.6%±18.

3.1.10 The effect of prostaglandins of the E series and seminal plasma on LAK activity

For many years there has been a great deal of interest in the immunosuppressive activities of prostaglandins, particularly those of the E series. Prostaglandins are known to be secreted by a variety of tumours, both *in vitro* and *in vivo*. Furthermore, human seminal plasma contains particularly high levels of PGE_2, a fact which is of interest when considered in the light of conditions such as retrograde ejaculation (Quayle 1988, 1989).

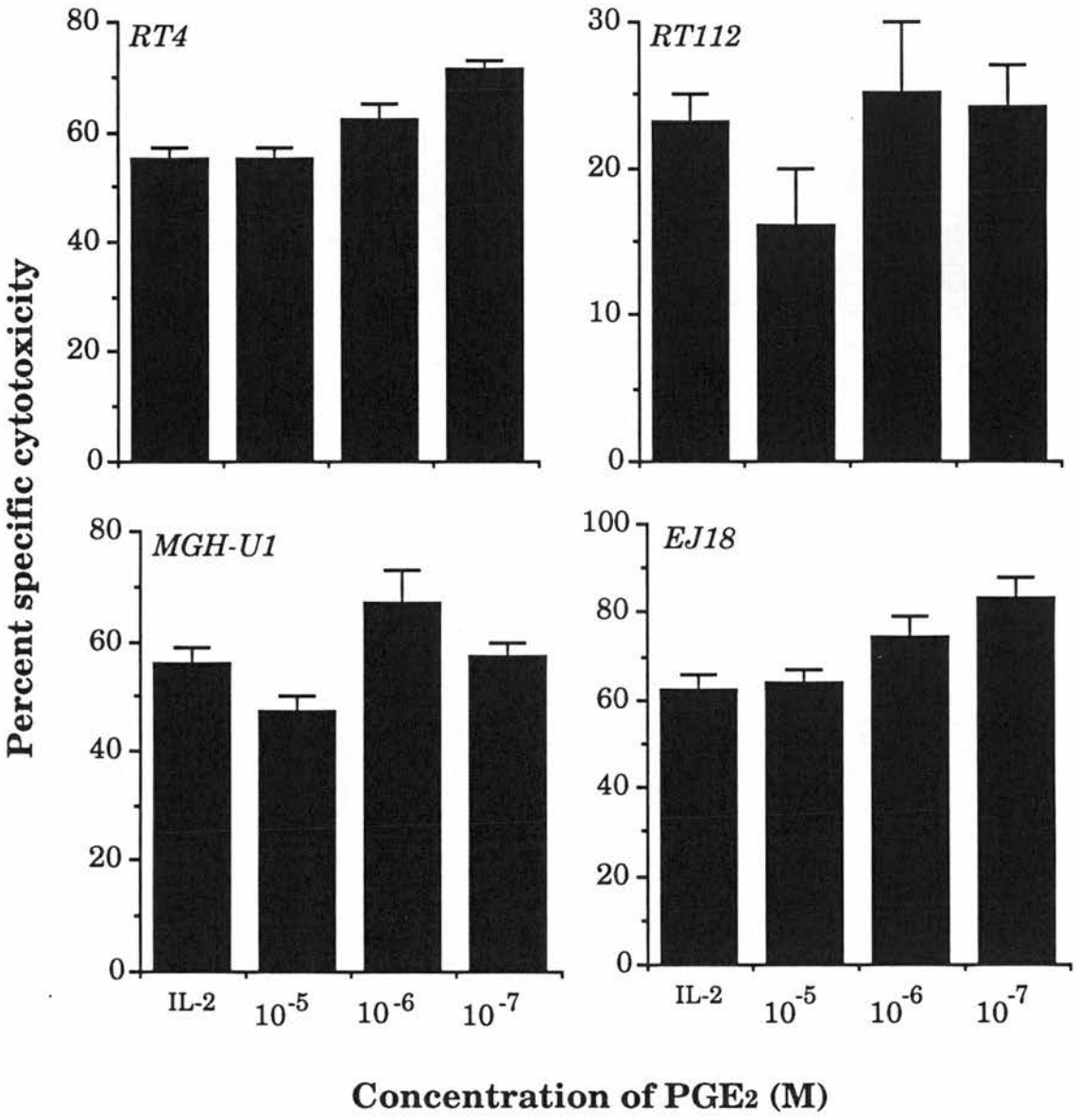
The effect of PGE1 and PGE2 on the generation and subsequent killing potential of LAK cells was investigated by activating cells in the presence of physiologically relevant concentrations of prostaglandin. Furthermore, cells were also activated in the presence of seminal plasma (known to contain high levels of PGE). When LAK cells were generated with 1,000Uml^{-1} IL-2 and between 10^{-5} and 10^{-7}M of PGE no suppression

Figure 15. Autologous LAK activity against fresh tumour cells



The ability of autologous LAK cells to kill freshly derived tumour cells. LAK cells were generated from patients PBMC by culture with 1,000U/ml IL-2 for 6 days in autologous serum. Target cells were isolated from tumour material obtained by cystoscopic resection of tumour mass. Cytotoxicity was determined using a standard chromium release assay and the results shown are those for seven patients. The standard deviation was within 5% of the experimental value.

Figure 16. The effect of PGE2 on the generation of LAK activity



Freshly isolated PBMN were placed in culture with 1,000U/ml recombinant human IL-2 for 6 days. In addition some cultures were also treated with the indicated concentrations of PGE2 (10^{-5}- 10^{-7} mM). Following incubation, the LAK activity of all cultures was examined using a standard cytotoxicity assay against four bladder cancer cell lines. The results represent the mean of triplicate determinations, error bars indicate the levels of 1 standard deviation.

of activity to levels lower than those achieved with IL-2 alone was observed (Figure 16). At the highest concentrations of PGE2 a decrease in activity against RT4 and EJ18 was observed, however this failed to reach significance ($p>0.5$). The results shown represent a single experiment. The experiment was repeated three times by C. Harrison, and similar results were obtained.

3.1.11 The endogenous production of cytokines by LAK cells

A variety of cytokines have been found to be secreted from cultures of PBL following activation with IL-2; Such cytokines include IFNγ and TNFα. The production of IFNγ and TNFα was examined using sensitive ELISA kit assay systems. Following culture of PBMC in the presence of IL-2 high levels of IFNγ and TNFα were detected. Levels of 100pg per 10^6 cells and and 50U per 10^6 cells of TNFα and IFNγ respectively were detected in tissues culture supernatants. In the absence of IL-2 only low levels of TNFα were detected (5pg per 10^6 cells). Spontaneous production of IFNγ was not observed. The function of such cytokines will be addressed in chapter *3.2.16.*

3.2 The expression and modulation of adhesion molecules

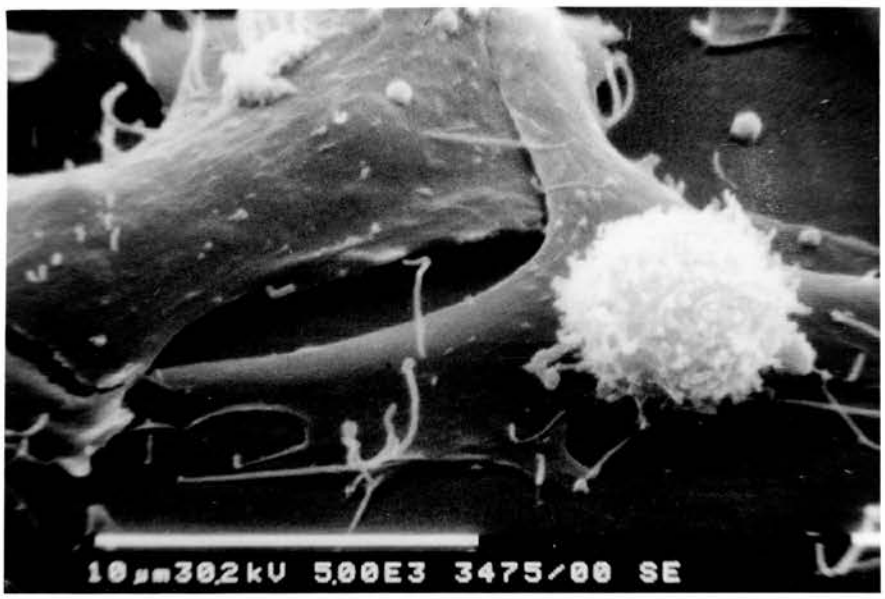
3.2.1 Scanning electron micrography of tumour and LAK cells

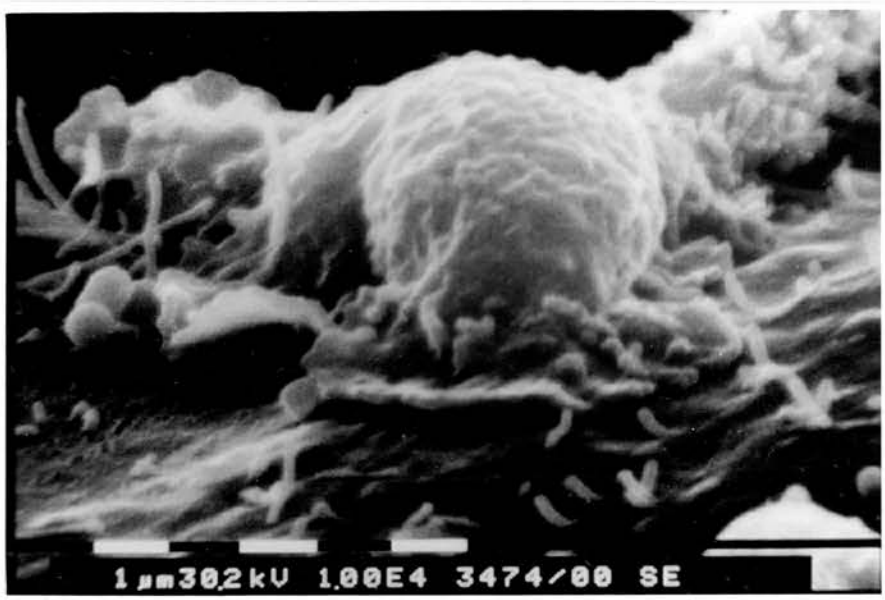
The mechanism of action involved in LAK mediated cytotoxicity remains undetermined although several theories have been postulated. It is known that several cytokines which are produced by LAK cells, notably interferon-gamma, can have direct anti-tumour effects on bladder cancer cells leading to cessation of proliferation and /or cytotoxicity. However, in order for these effects to be mediated prolonged exposure to these cytokines is required (>24 hours). It is therefore unlikely that humorally mediated events are responsible for killing by LAK cells. A more likely explanation would involve intimate contact between the effector and target cells, similar to that observed in killing by cytotoxic T-cells.

Using scanning electron micrography (SEM) we attempted to observe the intimate relationship between LAK cells and bladder cancer cells. Cultured tumour cells were incubated with LAK cells for 30 minutes and then processed for examination using SEM. Plate 1 shows two large, flat, high grade tumour cells, on top of which there is a LAK cell. When viewed from a lower angle (15°) the point of contact between the cells was observed (Plate 2). This appeared to take the form of a flat structure emanating from the LAK cell. The intimate nature of the relationship was confirmed when a LAK cells was pulled away from its target (Plate 3). Between the two cells, structures were evident and part of the LAK cell remained behind on the target.

The observation of intimate and firm contact between the effector and target cell prompted the search for adhesion molecules expressed by the effector and target cells which could account for such activity.

Plates 1, 2, and 3





3.2.2 The expression of adhesion molecules by bladder cancer cell lines

Using flow cytometry with monoclonal antibody probes the panel of TCC cell lines was examined for the constitutive expression of a variety of adhesion molecules (HLA class I, HLA class II, CD2, CD3, CD4, CD8, CD56 (NCAM), ICAM-1 (CD54), ICAM-2 and VCAM). The results are summarized in Table 5. All cell lines expressed HLA class I molecules but only RT4 (G1) expressed HLA class II. Expression of HLA class II on unactivated epithelial cells is unusual (personal communication, W. Bodmer). In order to confirm the existence of HLA class II a variety of monoclonal antibodies were used. Other than HLA molecules, the only other adhesion molecules expressed by TCC cells were members of the intercellular adhesion molecule family (ICAM). All eight cell lines constitutively expressed either ICAM-1 or ICAM-2, however, no cell line constitutively co-expressed both molecules. Cell lines of the lowest grade (G1) did not express ICAM-1, rather they expressed ICAM-2. The poorly differentiated high grade cell lines expressed ICAM-1 and not ICAM-2.

The percentage of cells which constitutively expressed either ICAM molecule varied between the different cell lines (Figure 17). Only a small percentage of RT4 and RT112 cells expressed ICAM-2 and ICAM-1 respectively whilst over 60% of EJ18 and J82 cells expressed ICAM-1.

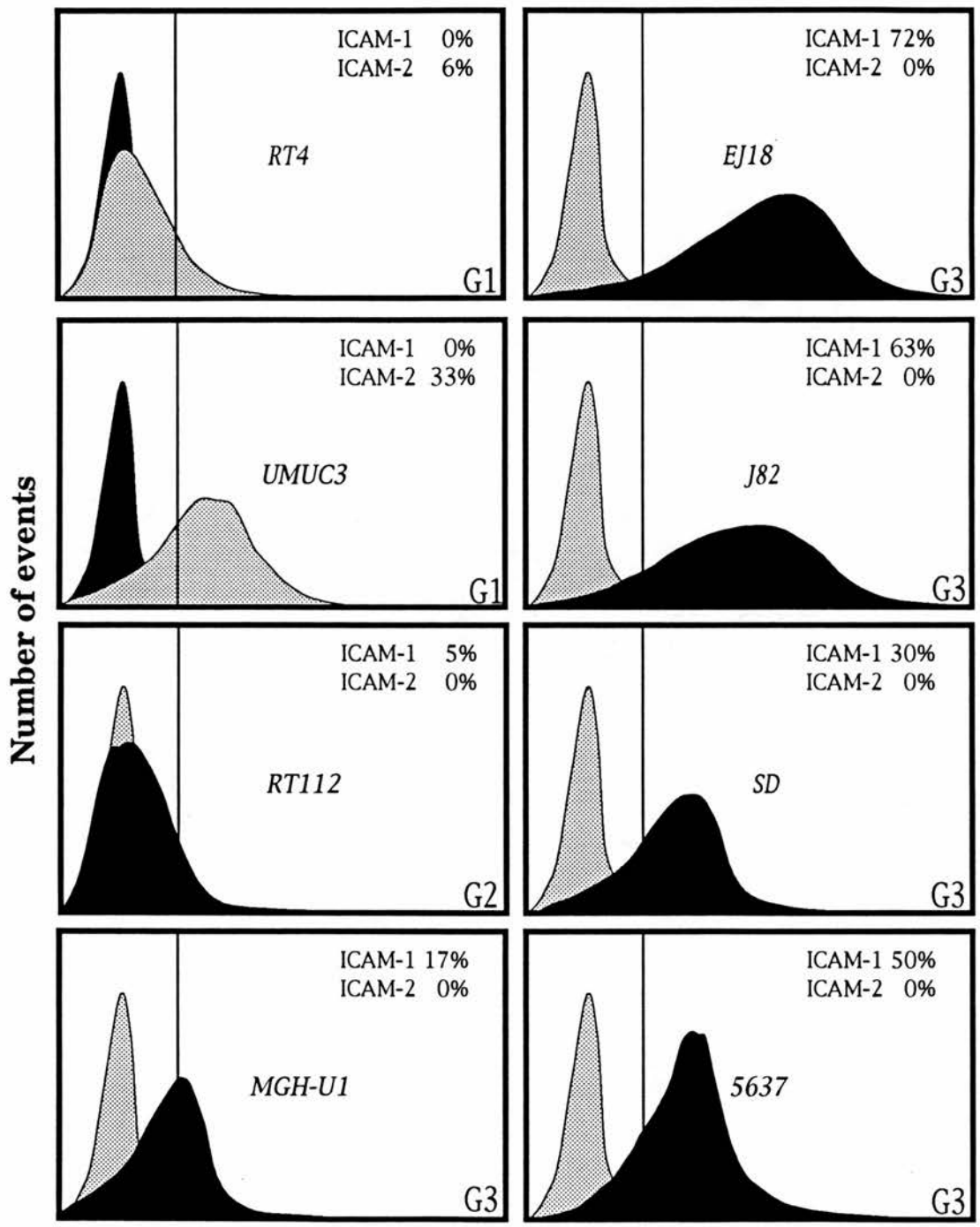
One major mechanism by which leucocytes adhere to vascular endothelium is via the cytokine inducible VCAM molecule. This molecule was a candidate for the ligand on tumour cells as VLA-4 is known to be expressed by lymphocytes. However, none of the cell lines constitutively expressed VCAM.

Table 5. The constitutive expression of adhesion molecules by TCC cell lines

Cell line	RT4	UMUC3	RT112	MGH-U1	EJ18	J82	SD	5637
Grade	G1	G1	G2	G3	G3	G3	G3	G3
HLA class I	++++	++++	++++	++++	++++	++++	++++	++++
HLA class II	+	-	-	-	-	-	-	-
CD2	-	-	-	-	-	-	-	-
CD3	-	-	-	-	-	-	-	-
CD4	-	-	-	-	-	-	-	-
CD8	-	-	-	-	-	-	-	-
CD56	-	-	-	-	-	-	-	-
ICAM-1	-	-	+	++	++++	++++	+++	+++
ICAM-2	+	++	-	-	-	-	-	-
VCAM	-	-	-	-	-	-	-	-

A summary of the constitutive expression of adhesion molecules by eight bladder cancer cell lines. Expression was determined using flow cytometry with monoclonal antibody probes. Also shown is the grade of the parent tumour from which the cell lines were derived and are still representative. No expression (-), ,10% of cells expressing (+), between 10 and 30% of cells expressing (++), between 30 and 50 % of cell expressing (+++), over 50 % of cells expressing the antigen of interest (++++).

Figure 17. Representation of the flow cytometric profiles of ICAM-1 and ICAM-2 expression by TCC cell lines



The constitutive expression of ICAM-1 and ICAM-2 by eight bladder cancer cell lines as studied by flow cytometry. Solid curves show ICAM-1 expression whilst shaded curves show the expression of ICAM-2. The vertical dividing line on each profile indicates the level of non-specific fluorescence.

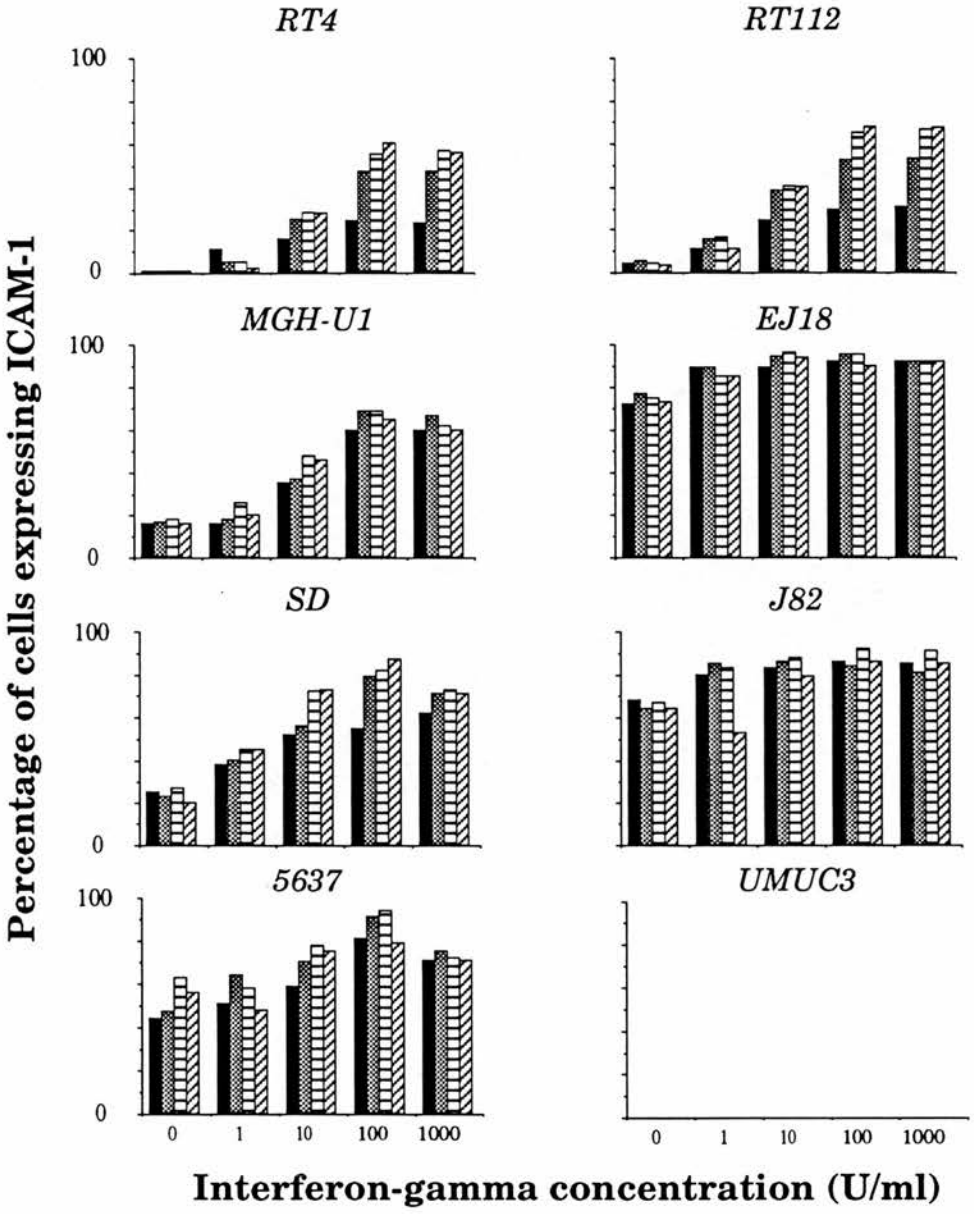
Previous work has demonstrated that IFNγ can induce and augment the expression of HLA class I and II molecules on the surface of bladder cancer cell lines *in vitro*. As ICAM-1 is an inducible molecule, the effect on ICAM-1 expression of various cytokines, some of which have been identified in the urine of patients receiving BCG therapy, by TCC cell lines was investigated.

3.2.3 The modulation of ICAM-1 expression by interferon-gamma

Bladder cancer cells were incubated with increasing doses of IFNγ for 0-48 hours and the expression of ICAM-1 was determined by flow-cytometry. ICAM-1 expression was induced or augmented on all the cell lines with the exception of UMUC-3 (G3) (Figure 18). The increased expression of ICAM-1 was dependent upon the concentration of IFNγ and upon the duration of the stimulus. Optimal induction was achieved using $100Uml^{-1}$ IFNγ after 24 hours of continuous stimulation. Further increases in the concentration and duration of stimulus failed to result in a corresponding increase in ICAM-1 expression. Rather, expression was decreased below maximal levels (SD and 5637). No ICAM-1 expression could be induced on the UMUC-3 cell line even with IFNγ concentration of $2,000Uml^{-1}$ and exposure periods in excess of 200 hours (data not shown). The expression of ICAM-2 on all cells lines was not affected by stimulation with IFNγ (data not shown).

When the panel of bladder cancer cell lines were optimally stimulated for the expression of ICAM-1 there was a marked difference in the percentage of cells which were deemed positive. Only 58%±2.4 of optimally stimulated RT4 cells as compared to 97%±2 of EJ18 or 93%±3.6 of SD cells expressed ICAM-1.

Figure 18. Interferon-gamma increases ICAM-1 expression



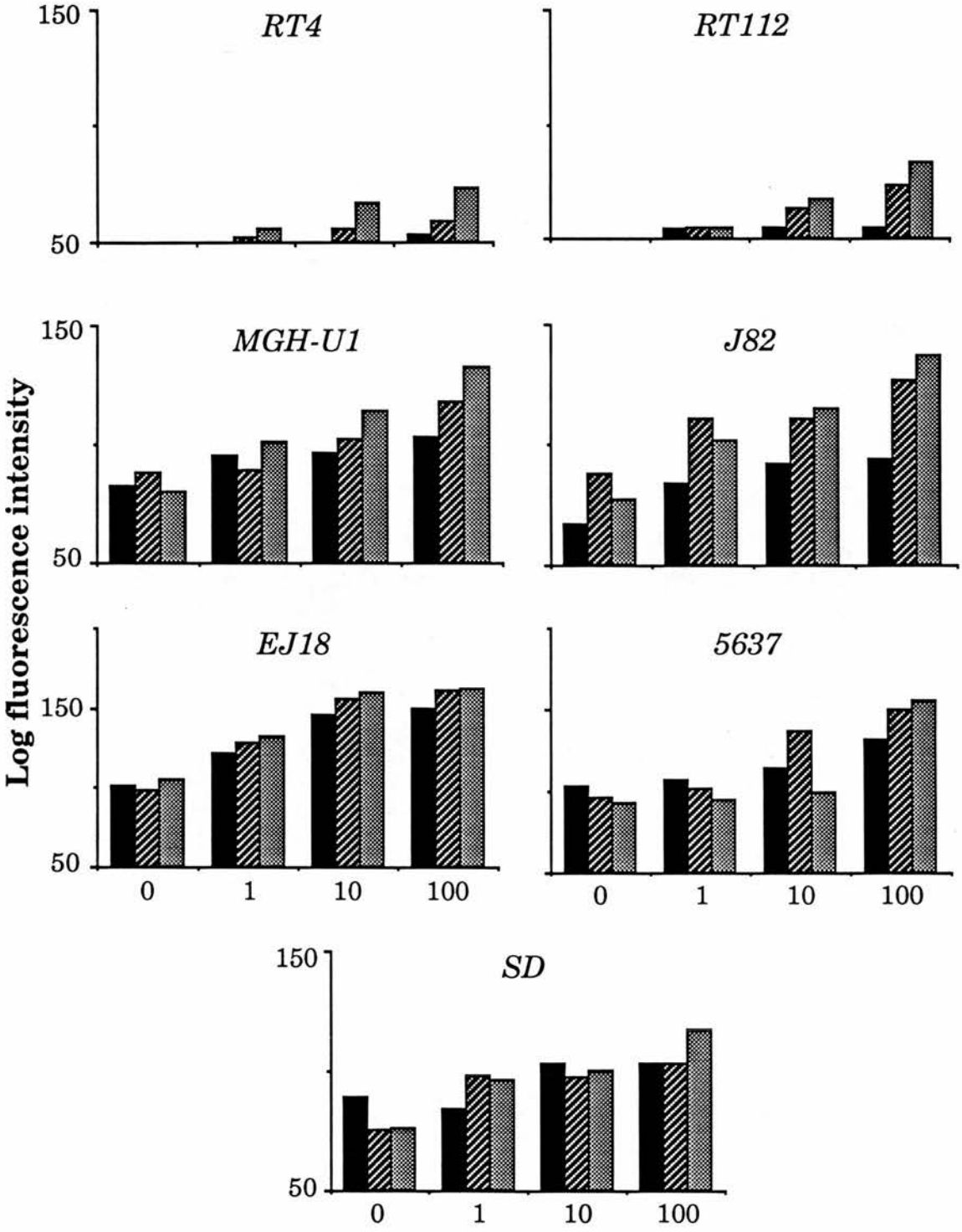
The effect of stimulation with IFNγ on ICAM-1 expression by bladder cancer cell lines. TCC cell lines were stimulated for 6 (■), 12 (▩), 24 (⊟), and 48 (▨) hours with the indicated concentrations of IFNγ. ICAM-1 expression was determined by flow cytometry. Bars represent the mean of triplicates from a representative experiment. All standard deviations were within 5 % of experimental values.

When TCC cell lines were stimulated with IFNγ not only was there an increase in the percentage of cells expressing ICAM-1 but there was an increase in the intensity of the ICAM-1 staining. For all cell lines which expressed ICAM-1 following stimulation the maximum antigen density was observed with $100Uml^{-1}$ after 24 hours stimulation (Figure 19). Increases in the intensity of antigen staining may be due to a change in the volume of the cell, however, no such change was observed during these experiments (data not shown). An increase in the density of ICAM-1 expressed by a target cell may be important as both the affinity and the avidity of the LFA-1/ICAM-1 interaction are important in determining the extent of LFA-1 dependent cytotoxicity.

The effect of continual stimulation with IFNγ on ICAM-1 expression is show in Figure 20. Induction was rapid with significant increases in ICAM-1 expression evident after as little as 4 hours stimulation ($p<0.02$). Optimal induction occurred after 24 hours, following which a steady decline in the level of ICAM-1 was observed.

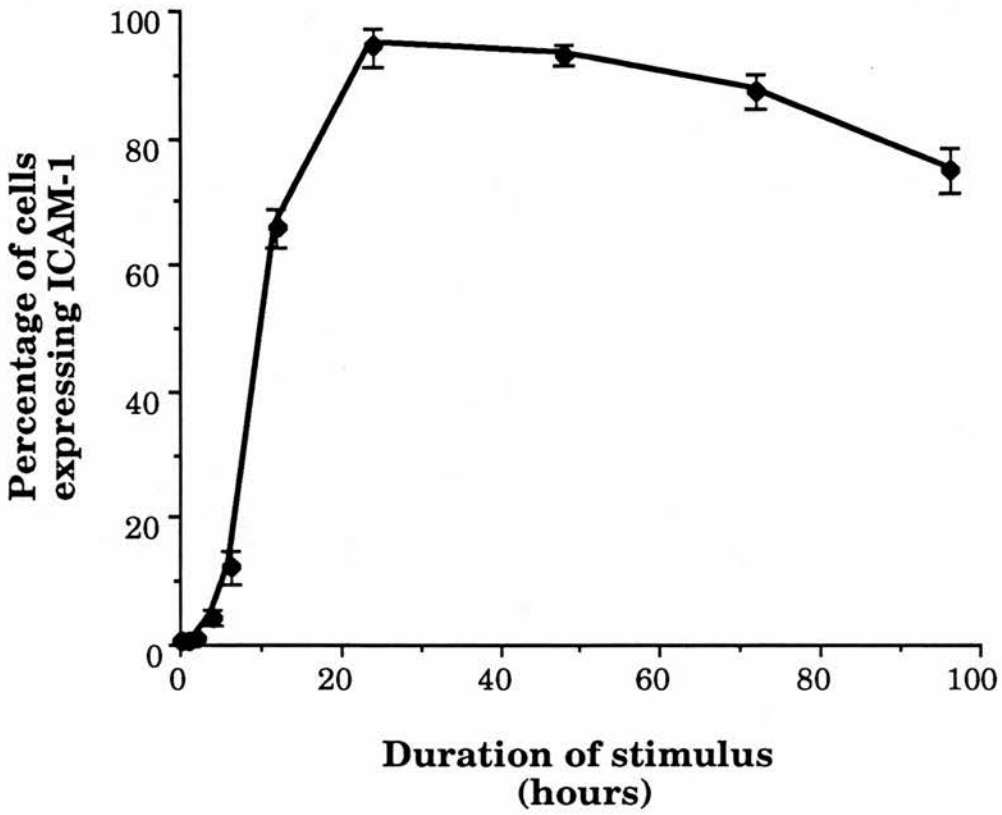
Following BCG therapy, IFNγ is transiently produced and secreted into the urine in large quantities. Therefore, bladder cancer cells may only be exposed to IFNγ for a short and limited period of time. As has been shown above, the induction of ICAM-1 expression by IFNγ is a rapid event, the earliest signs of which were observed 4 hours after stimulation. The minimum duration of continuous stimulation required to generate a response was investigated. When cells were stimulated with IFNγ for 10 seconds, washed repeatedly, and then cultured for the remainder of 24 hours, a significant increase in ICAM-1 expression was observed ($p<0.02$) (Figure 21). Longer periods of stimulation, up to 24 hours continuous stimulation, resulted in increased ICAM-1 expression. Following 12 hours stimulation (the duration received by cells in the bladder following BCG

Figure 19. Interferon-gamma increases the density of ICAM-1



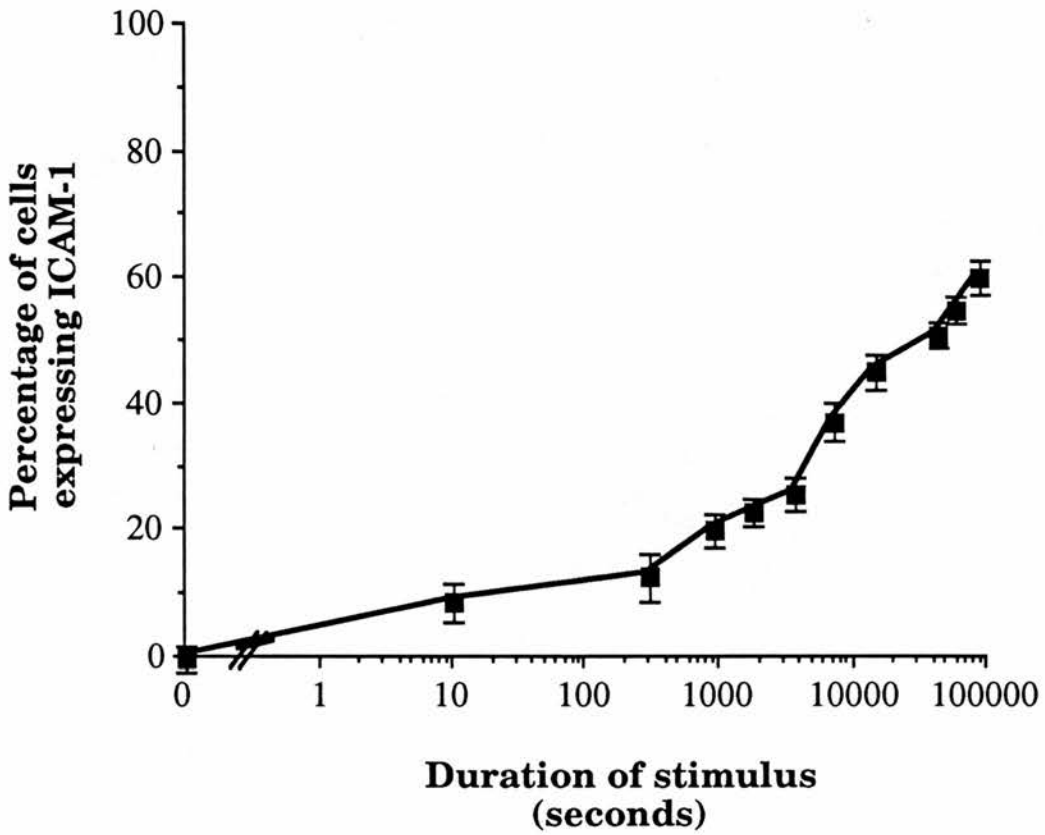
The effect of IFNγ stimulation on the intensity of ICAM-1 expression by bladder cancer cell lines. Cells were incubated with the indicated concentrations of IFNγ for 6 (■), 12 (▨) , and 24 (▩) hours. ICAM-1 expression was determined using flow cytometry. Bars represent the mean of triplicate determinations.

Figure 20. The effect of constant stimulation with interferon-gamma on ICAM-1 expression



The time-course of induction of *de novo* ICAM-1 expression by RT4 following stimulation with 100U/ml IFNγ. ICAM-1 expression was determined using flow cytometry. Error bars represent 1 standard deviation.

Figure 21. The duration of stimulus required for ICAM-1 induction



The dependence of ICAM-1 expression by RT4 upon duration of stimulus. Cells were exposed to 100U/ml of IFNγ for the indicated duration following which, the IFNγ was removed and the cells washed three times in order to remove the remaining IFNγ. The cells were then cultured for a total time of 24 hours from the onset of stimulation. ICAM-1 expression was determined using flow cytometry. Error bars indicate 1 sd.

therapy), over 80% of the maximal response had been achieved.

As mentioned, the bladder cancer cell lines in this study failed to constitutively express VCAM. This observation was extended by studying the expression of VCAM following stimulation with IFNγ. The cell line MGH-U1 was stimulated with a range of concentration of IFNγ for 0-48 hours following which the expression of VCAM was studied. No cells were seen to express VCAM following such stimulation (data not shown). It therefore seem unlikely that VCAM /VLA-4 interactions are involved in the LAK mediated cytolysis of bladder carcinoma cell lines.

3.2.4 Tumour necrosis factor-alpha modulates ICAM-1 expression

As TNFα is readily detected in the urine of patients who received BCG therapy for superficial bladder cancer, its effect on ICAM-1 expression by TCC cell lines was investigated. When cells were stimulated with TNFα for 24 hours the ICAM-1 expression on such cells was increased (Figure 22). Optimal induction was achieved with $100Uml^{-1}$ of TNFα for all four cell lines tested. The ICAM-1 inducing effects of TNFα were of smaller magnitude than those observed following stimulation with IFNγ.

3.2.5 Interleukin-1 modulates the expression of ICAM-1

Interleukin-1, a product of activated T-cells and macrophages, is detected in the urine of patients following repeated instillations of BCG vaccine. This cytokine has been shown to induce ICAM-1 expression on vascular endothelium. Its ability to increase ICAM-1 expression on the surface of bladder cancer cells was therefore studied.

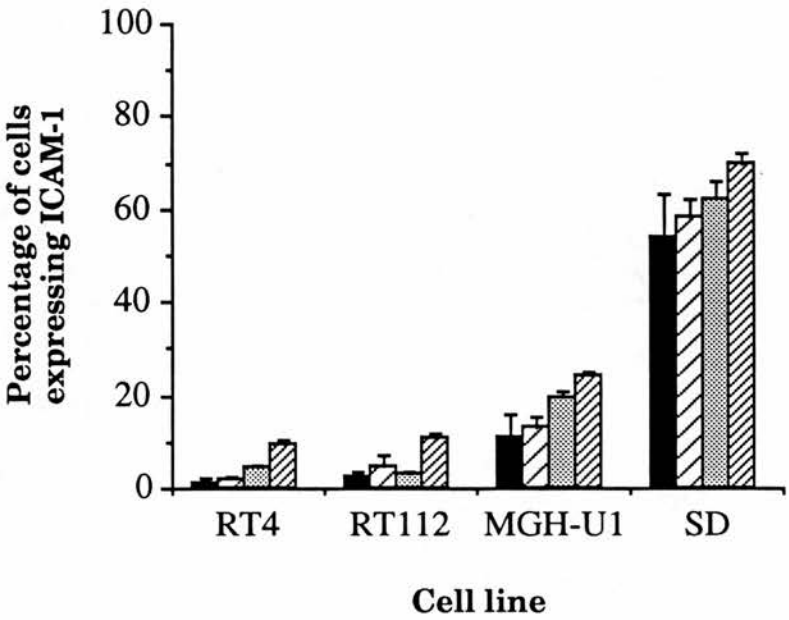
Stimulation of TCC cell lines with IL-1α for 24 hours elicited a dose dependent response of increased ICAM-1 expression (Figure 23). Optimal induction occurred when cells were stimulated with either 100Uml^{-1} (MGH-U1) or 1,000Uml^{-1} (RT4). In the case of MGH-U1 the effects of IL-1α were comparable to those of IFNγ, however, for all the other cell lines tested the effects of IL-1α on ICAM-1 expression were far smaller than those achieved using IFNγ.

3.2.6 The effect of other cytokines on ICAM-1 expression by TCC cell lines

A variety of cytokines other than IFNγ, IL-1 and TNFα are detected in the urine following BCG therapy. These include IL-2, a cytokine produced by activated T-cells and in part responsible for the generation of LAK cells.

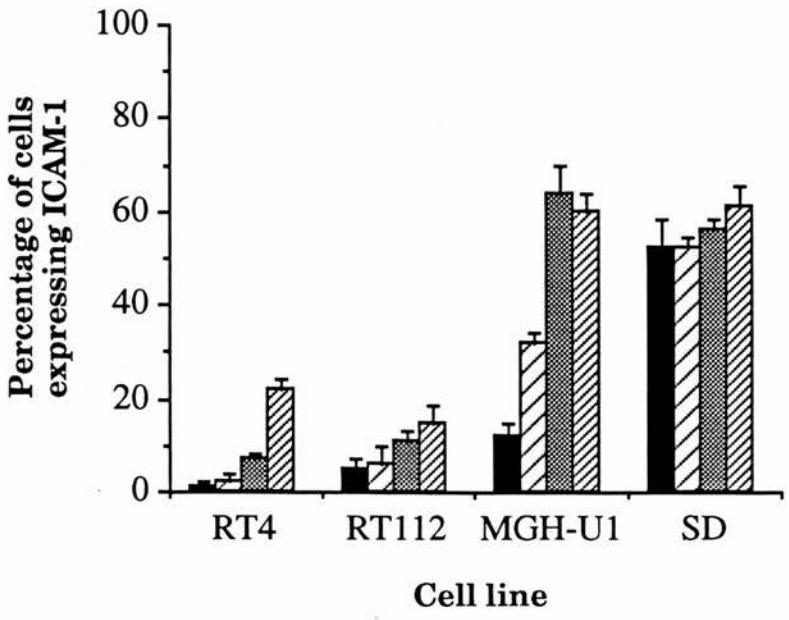
Bladder cancer cells were stimulated with a variety of cytokines for 24 hours prior to determination of their ICAM-1 expression. The cytokines IL-2, IL-3, IL-4, IL-7, IL-8, TGFß and GM-CSF did not modulate the constitutive expression of ICAM-1 on any of the bladder cancer cell lines tested (Figure 24). No changes were observed in either the percentage of cells positive or in the density of the antigen on the cell surface (data not shown). Although these cytokines did not appear to affect the expression of the ICAM-1 gene, this does not necessarily preclude them from exhibiting other effects on the target cells (discussed later).

Figure 22. Tumour necrosis factor-alpha induces ICAM-1 expression



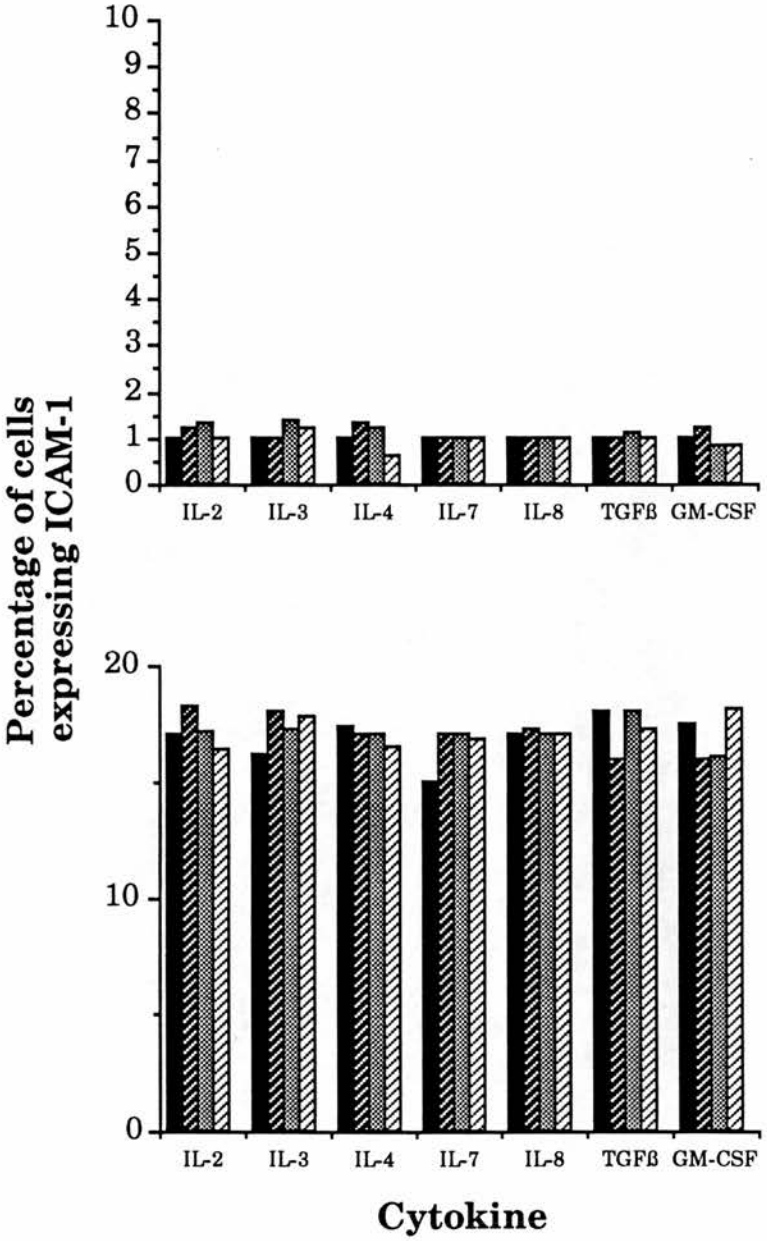
The effect of TNFα stimulation on the expression of ICAM-1 by four TCC cell lines. Cells were exposed to 0 (■), 1 (▨), 10 (▩), and 100 (▨) U/ml of TNFα for 24 hours. ICAM-1 expression was determined by flow cytometry.

Figure 23. Interleukin-1-alpha induces ICAM-1 expression



The effect of IL-1α stimulation on the expression of ICAM-1 by four TCC cell lines. Cells were exposed to 0 (■), 1 (▨), 10 (▩), and 100 (▨) U/ml of IL-1α for 24 hours. ICAM-1 expression was determined by flow cytometry. Error bars represent 1 standard deviation.

Figure 24. Other cytokines do not modulate ICAM-1 expression



The modulation of ICAM-1 expression by cytokines other than IFNγ, TNFα or IL-1α. TCC cell lines were incubated with 0 (■), 10 (▨), 100 (▩) and 1000 (▨) U/ml of the indicated recombinant human cytokines for 24 hours. ICAM-1 expression was identified using flow cytometry with monoclonal antibody probes. The bars represent the mean of triplicates, standard deviations were within 10% of the experimental values. The upper figure shows the response of RT4 cells and the lower the response of MGH-U1 cells.

3.2.7 Interferon-alpha-2a does not modulate constitutive ICAM-1 expression but suppresses IFNγ induced ICAM-1 expression

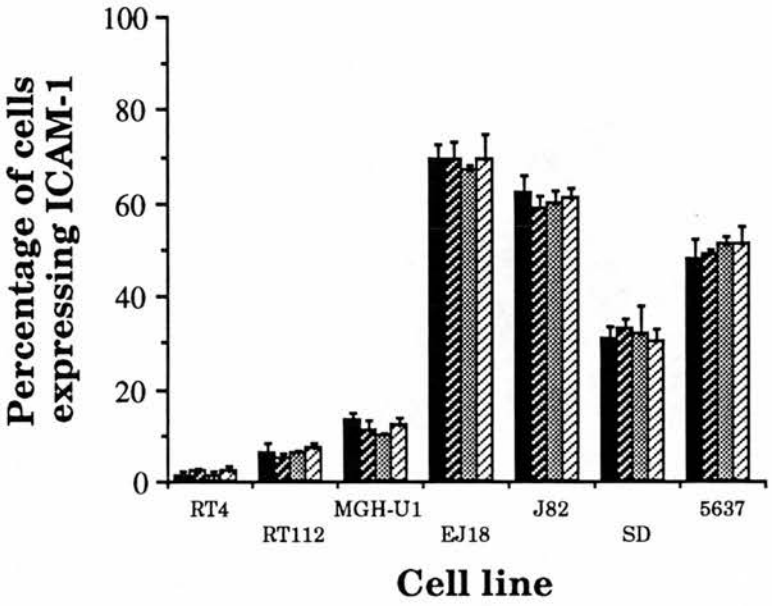
When cells were stimulated with IFNα no change in the constitutive expression of ICAM-1 was observed (Figure 25). However, simultaneous stimulation with IFNγ and IFNα resulted in decreased ICAM-1 expression as compared to that generated by stimulation with IFNγ alone (Figure 26). The decrease in ICAM-1 expression was dose dependent with optimum activity occurring with $100Uml^{-1}$ of IFNα. The expression of ICAM-1 by EJ18 was not reduced by simultaneous stimulation with IFNα.

3.2.8 Interferon-gamma synergizes with TNFα in the upregulation of ICAM-1 expression

It has been demonstrated that IFNγ and TNFα or IL-1 synergize in the regulation of ICAM-1 expression on a variety of cell types. As both molecules have been detected in the urine of patients receiving BCG therapy, any possible synergistic actions were investigated.

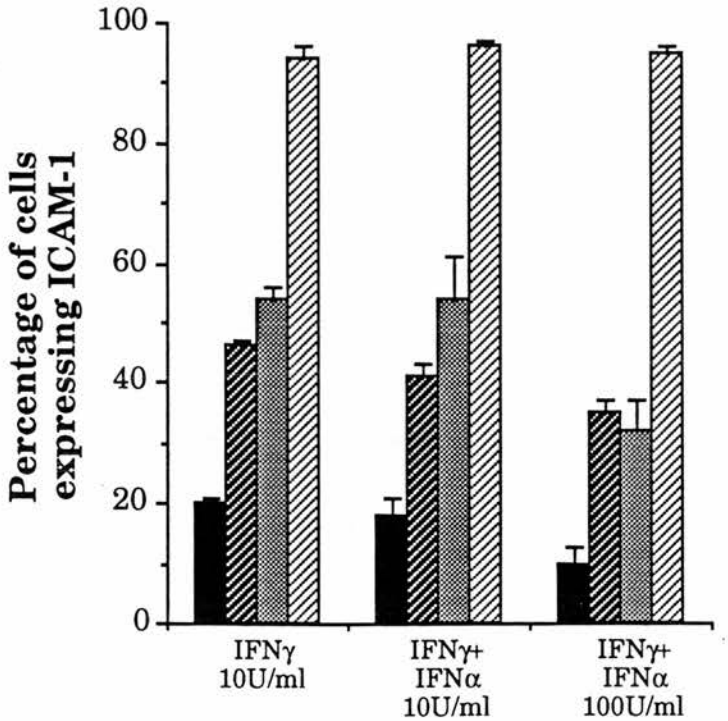
When bladder cancer cell lines were stimulated with a combination of IFNγ and TNFα a greater response was seen than that generated with a comparable concentration of either molecule singly (Figure 27). Furthermore, the effect was greater than additive thus demonstrating synergistic up-regulation of ICAM-1 expression. The synergy between the two cytokines was greatest when stimulated with $10Uml^{-1}$ of each for 24 hours. This concentration is of the same order as that produced in response to BCG therapy (see section *3.6*).

Figure 25. Interferon-α does not modulate constitutive ICAM-1



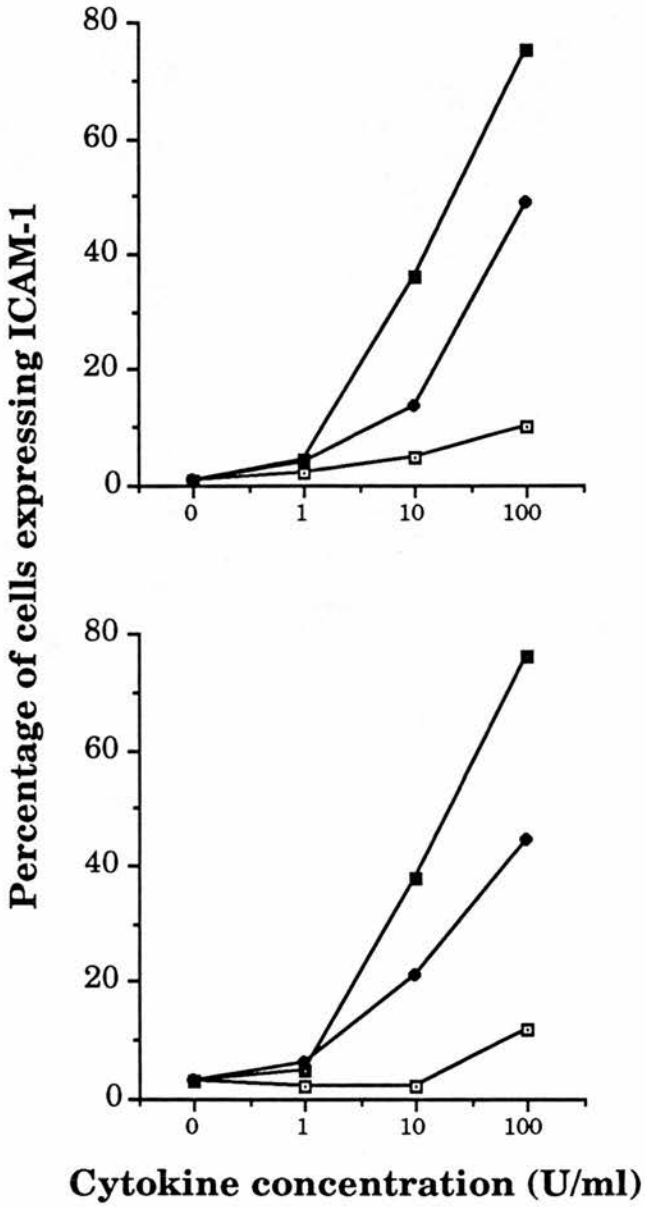
The effect of stimulating bladder cancer cell lines with recombinant human IFNα-2a. Cells were stimulated with 0 (■), 10 (▨), 100 (▩), and 1000 (▨) U/ml of IFNα for 24 hours. ICAM-1 expression was determined using flow cytometry. Bars represent the mean of triplicate determinations and error bars represent 1 standard deviation.

Figure 26. IFN-α decreases IFNγ induced ICAM-1



Cells were stimulated with IFNγ alone or in the presence of IFNα for 24 hours. The four cell lines used wer RT4 (■), RT112 (▨), MGH-U1 (▩), and EJ18 (▨). ICAM-1 expression was determined using flow cytometry.Bars represent the mean of triplicate determinations and error bars represent1 standard deviation.

Figure 27. The synergy between IFNγ and TNFα in ICAM-1 induction



Cells were stimulated for 24 hours with the indicated concentrations of either IFNγ (●), TNFα (▫), or both cytokines simultaneously (▪). The expression of ICAM-1 was studied using flow cytometry with monoclonal antibody RR1/1. The upper graph shows the results obtained with RT4 and the lower graph RT112. Standard deviations were within 10% of experimental values.

3.2.9 The effect of pulsed stimulation with IFNγ on ICAM-1 expression

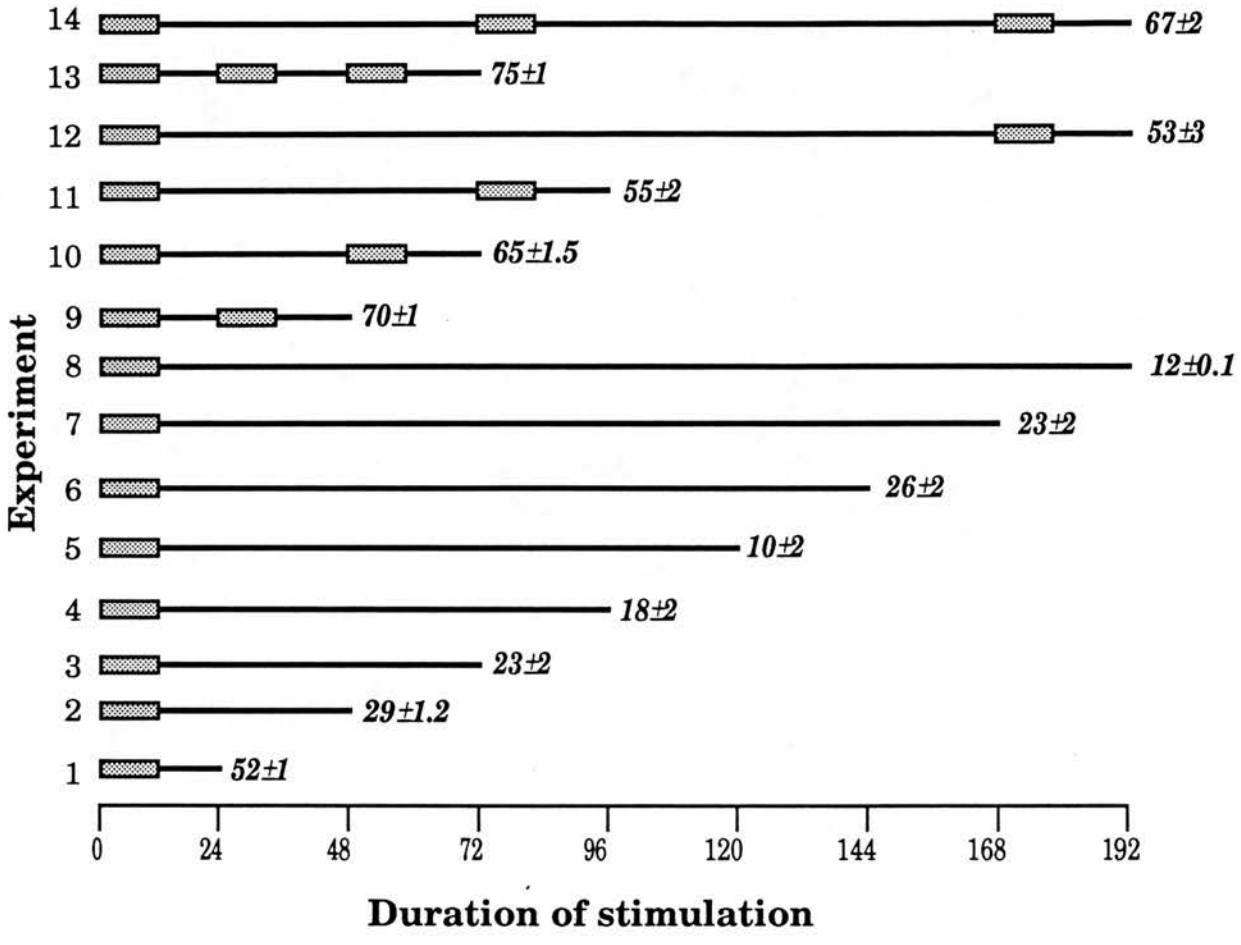
Following BCG treatment transient production (~12hrs.) of IFNγ has been observed (Prescott *et al* 1990). The effect of single and repeated 12 hour pulses of IFNγ was investigated in an attempt to mimic the *in vivo* situation of repeated stimulation.

Cells were firstly stimulated for 12 hours with $100Uml^{-1}$ IFNγ following which the cells were washed and the culture period continued for the desired length of time. Following a single 12 hour pulse, the time course of ICAM-1 expression was studied (Figure 28, experiments 1-8). Maximal expression was again observed after 24 hours, declining to low levels with 120 hours total culture period (expt. 5).

When two pulses of IFNγ were given, 12 hours apart, an increase in ICAM-1 expression was noted (expt. 9). If the second pulse was delayed for a further 24 or 48 hours, a lesser response was observed (expts. 10, and 11). Secondary stimulation after 72 hours failed to produce a response greater than control (expt. 12).

The highest response was obtained with three pulses, each 12 hours apart (expt. 13), however, a response greater than control was also obtained when the second and third pulse were given with 72 and 96 hour intervals respectively (expt. 14). Therefore, repeated stimulation with IFNγ at a point when ICAM-1 expression has not declined to baseline levels, yields an increase in expression greater than that maximally obtained following a single pulse.

Figure 28. Pulsed stimulation results in increased ICAM-1 expression



The expression of ICAM-1 molecules by RT4 following pulsed stimulation with IFNγ. Cells were exposed to 100U/ml of recombinant IFNγ for the indicated 12 hour periods (▨) following which they were washed with PBS three times and allowed to remain in culture for the duration of the stimulation (——). Shown is the percentage of cells expressing ICAM-1 as determined by flow cytometry, results are expressed as the mean ± 1 standard deviation.

3.2.10 Is there a role for mRNA pools in IFNγ induced ICAM-1 expression?

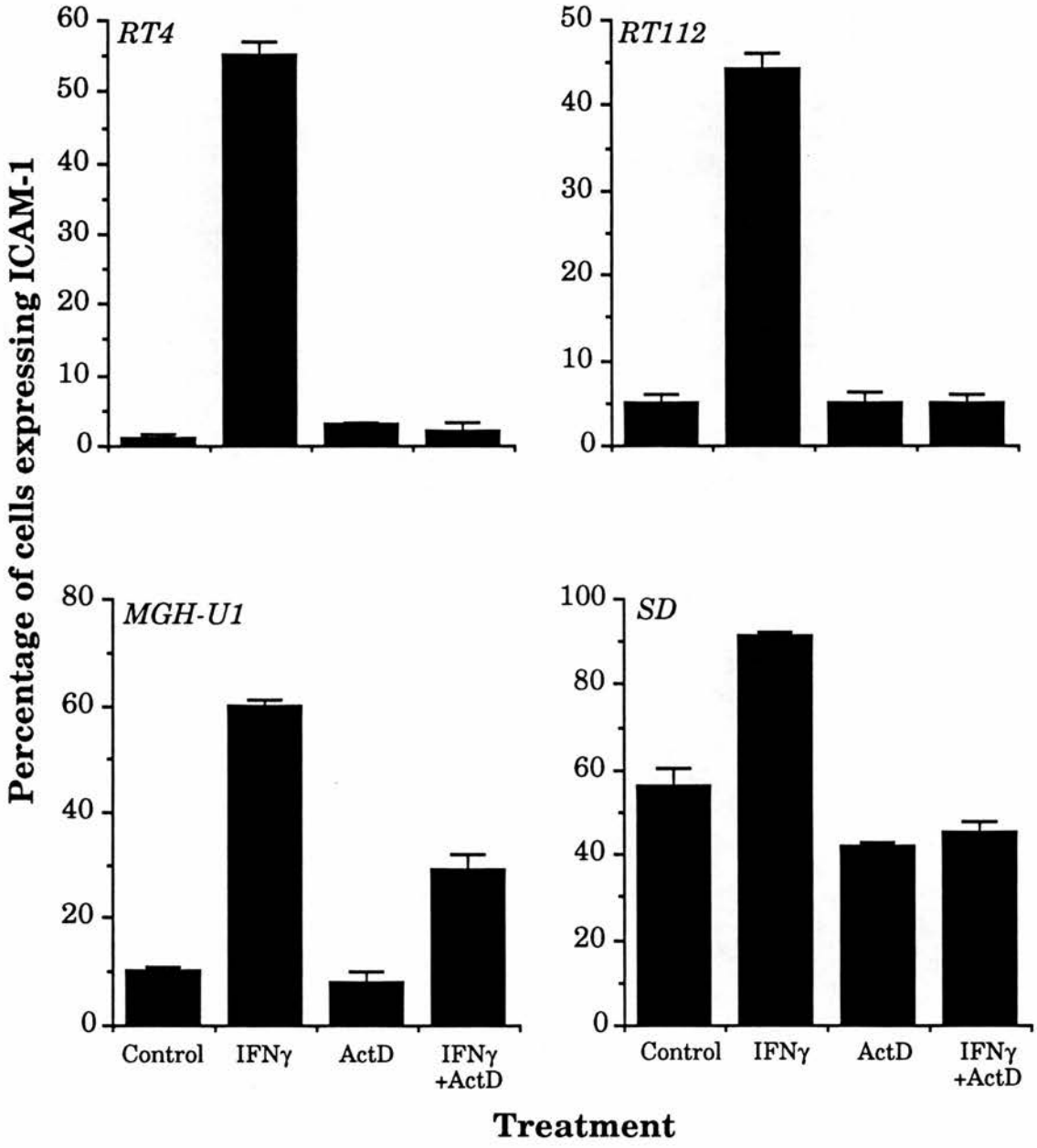
The above studies demonstrate the rapid appearance of membrane associated ICAM-1 following stimulation with IFNγ. One possible means by which ICAM-1 could be rapidly expressed is by the existence of intracellular pools of mRNA and/or protein. In order to investigate this possibility, we treated cells with actinomycin D (an inhibitor of mRNA transcription) prior to stimulation with IFNγ for 24 hours. Pre-treatment with actinomycin D ($1\mu gml^{-1}$ for 30 minutes) completely prevented IFNγ from inducing the expression of ICAM-1 on three of the four cell lines tested (Figure 29). When MGH-U1 was treated with actinomycin D and IFNγ, an increase in ICAM-1 expression was observed which was greater than control but less than that obtained with IFNγ alone.

3.2.11 Is there a role for intracellular protein pools in IFNγ induced ICAM-1 expression?

The inhibitor of protein synthesis cycloheximide (CHX) was used to prevent the production of new ICAM-1 molecules. Following treatment with CHX ($1\mu gml^{-1}$ for 30 minutes) the cells were stimulated with IFNγ in the presence of CHX. Pre-treatment with CHX prevented the induction of ICAM-1 expression by IFNγ in the cell lines RT4 and RT112 (Figure 30). However, when MGH-U1 cells were stimulated in an identical manner the levels of expression was marginally increased, rather than decreased.

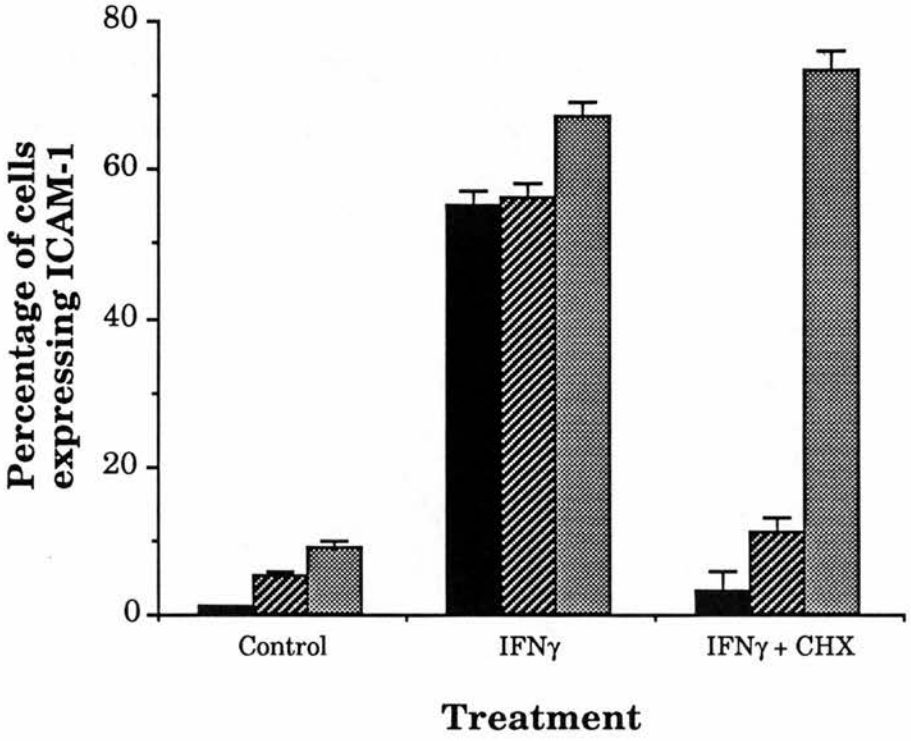
When cells were first stimulated for 24 hours with IFNγ, the IFNγ removed by repeated washing, and replaced by medium containing CHX, and the time course of ICAM-1 expression studied, interesting results were noted (Figure 31). Rather than the level of ICAM-1 expression remaining

Figure 29. There is no pool of untranslated mRNA for ICAM-1



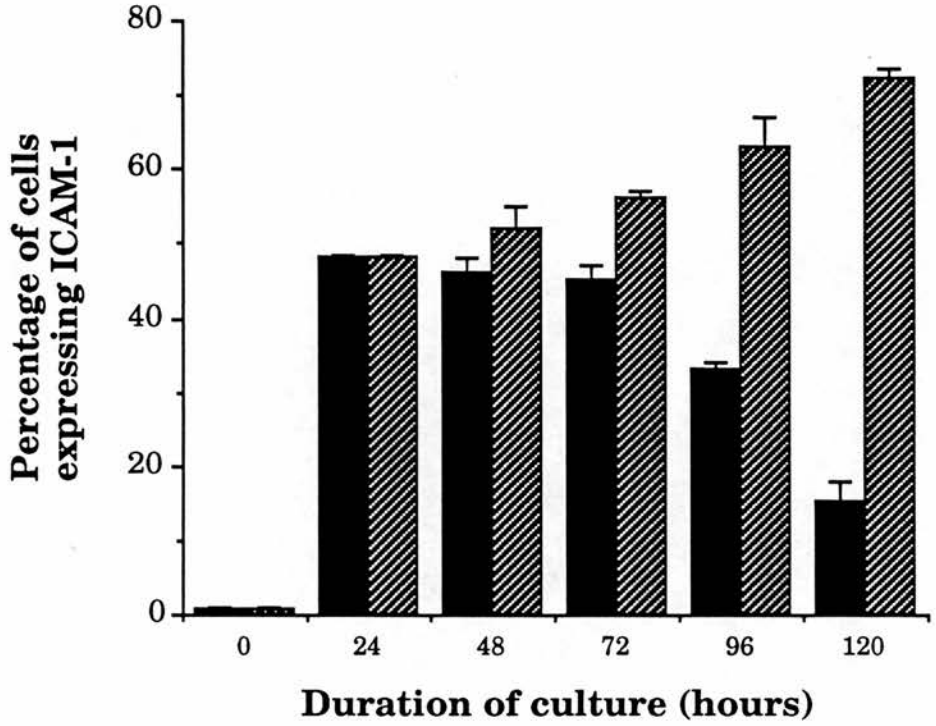
Four bladder cancer cell lines were stimulated with IFNγ (100U/ml), actinomycin D (1μg/ml) or both for 24 hours. The expression of ICAM-1 was determined by flow cytometry. The values shown represent the mean of triplicate determinations, error bars indicate 1 standard deviation.

Figure 30. The role of protein synthesis in IFNγ induced ICAM-1 expression



The contribution of protein synthesis to IFNγ induced ICAM-1 expression was investigated using the protein synthesis inhibitor cycloheximide (CHX). Three cell lines were used for this experiment; RT4 (■), RT112 (▨), and MGH-U1 (▩). Cells were cultured for 24 hours with no exogenous stimulus, IFNγ at 100 U/ml, or IFNγ and CHX at 1μg/ml . Following stimulation the expression of ICAM-1 was determined using flow cytometry with monoclonal antibody RR1/1. The bars represent the mean of triplicate determinations, error bars indicating the level of 1 standard deviation.

Figure 31. The inhibition of protein synthesis serves to stabilize cell surface ICAM-1 molecules



The effect of inhibition of protein synthesis on the IFNγ induced ICAM-1 expression by the cell line RT4. Cells were first stimulated for 24 hours with 100U/ml IFNγ following which they were cultured in the absence (■), or presence (▨) of CHX (1μg/ml) for the indicated period. Results represent the mean of triplicates, error bars showing 1 standard deviation.

constant, an increase in the percentage of cells expressing ICAM-1 was observed. Therefore, following stimulation maximal expression is observed by 24 hours, after which the level of expression falls gradually. However, it would appear that at 24 hours not all the ICAM-1 protein is expressed on the cell surface, rather it is located intracellularly. Furthermore, it would appear that CHX treatment serves to stabilize the ICAM-1 expressed on the cell surface, thus preventing recycling or shedding.

3.2.12 Regulation of ICAM-1 expression by retinoic acid

In addition to various cytokines, retinoic acid has also been reported to up-regulate the expression of ICAM-1 on a variety of tumour cells. The effect of retinoic acid on the expression of ICAM-1 by bladder cancer cells was therefore investigated. Two cell lines were stimulated with between 0.1 and 100μgml^{-1} of all-*trans* retinoic acid for between 24 and 96 hours, following which the expression of ICAM-1 was studied. Expression of ICAM-1 molecules on either RT4 or MGH-U1 was not increased by treatment with retinoic acid at any concentration within the time-course studied (data not shown). As a control, cells which were stimulated with IFNγ, and found to express elevated levels of ICAM-1 as expected.

3.2.13 Prostaglandins of the E series do not modulate ICAM-1 expression

The role of prostaglandins E1 and E2 in the regulation of molecules which were potentially of immunological significance was investigated. Neither PGE1 or PGE2 treatment of bladder cancer cell lines had any detectable effect on the constitutive expression of ICAM-1 (Figure 32). This was true for both the percentage of cell expressing ICAM-1 and for

the relative intensity of the staining (data not shown). Prostaglandins also failed to affect ICAM-1 expression as induced by IFNγ (Figure 33).

3.2.14 The expression of LFA-1 by IL-2 stimulated LAK cells

The ligand for the three ICAM molecules is LFA-1. As ICAM-1 and-2 were expressed by bladder tumour cells it was considered pertinent to examine the effector cells for the expression of LFA-1.

Using monoclonal antibodies to CD11a and CD18, freshly derived PBL were found to express high levels of both these antigens at two different densities. The mean levels of CD11a expression by lymphocytes and large granular lymphocytes was determined to be 93.8%±2, of CD18 expression 93.1%±2, of ICAM-1 34.6%±7.5, and of ICAM-2 73.9%±11. Following six days of activation with recombinant IL-2 no changes in the percentage of cells expressing the CD11a or CD18 molecules was observed. It was noticed that both CD11a and CD18 antigens were expressed at two different densities. The high density expression was found to be due to LGL, the lower density to lymphocytes.

3.2.15 The secretion of soluble ICAM-1 molecules by bladder cancer cell lines

When associated with the cell surface, ICAM-1 is a major ligand for LFA-1 and its associated effector functions. However, in a soluble form ICAM-1 has been shown to inhibit several LFA-1 dependent processes (Becker *et al* 1991). Following stimulation with IFNγ bladder cancer cell lines express high levels of cell surface ICAM-1. However, after 24 hours of stimulation the levels of cell surface ICAM-1 declines. The fate of such

Figure 32. Prostaglandins do not alter constitutive ICAM-1 expression

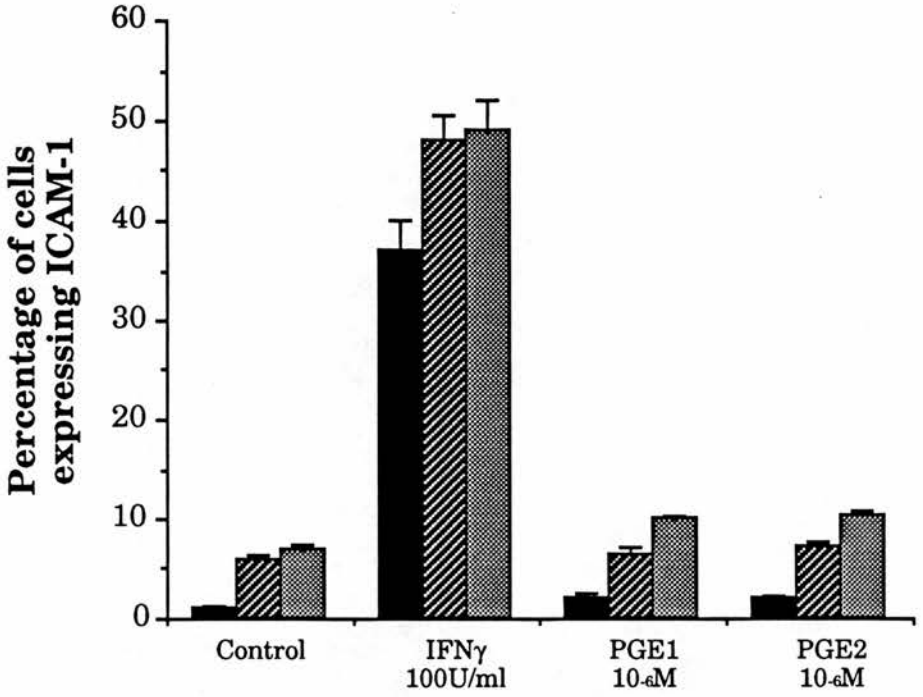
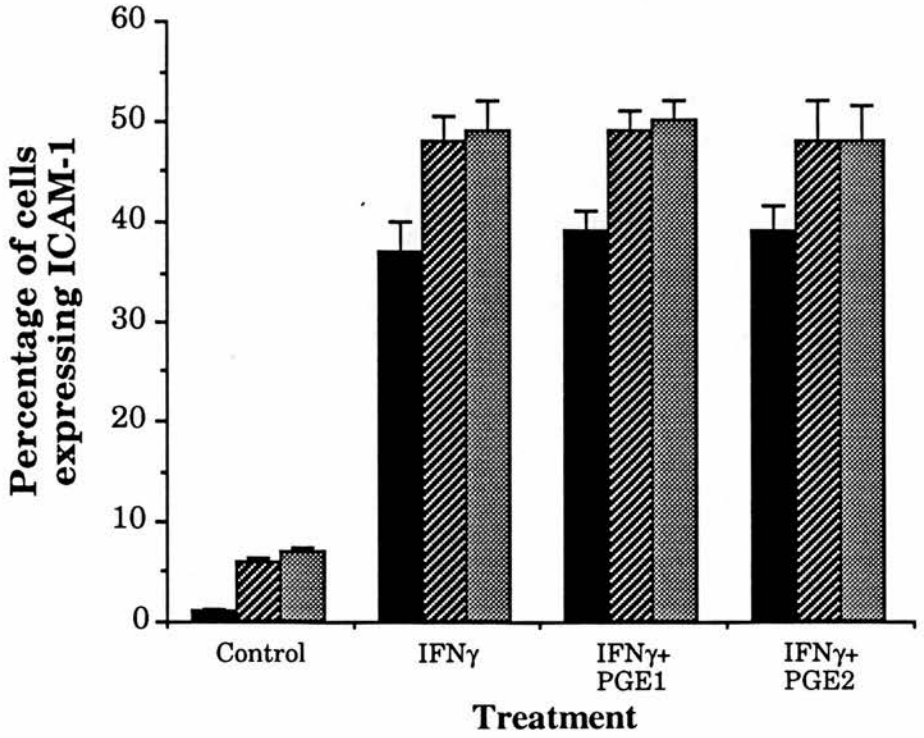


Figure 33. Prostaglandins do not alter IFNγ induced ICAM-1 expression



The effect of prostaglandins of the E series on the expression of ICAM-1 by 3 bladder cancer cell lines; RT4 (■), RT112 (▨), and MGH-U1 (▩). Cells were stimulated with 100U/ml of IFNγ either alone or in the presence of 10^{-6}M PGE1 or 2 for 24 hours prior to determination of ICAM-1 expression by flow cytometry. The results show a representative experiment in triplicate, error bars indicating 1 standard deviation.

ICAM-1 was investigated by studying the production of sICAM-1 by bladder cancer cell lines with the aid of a commercially available immunoassay kit.

Constitutively, the four cell lines investigated did not produce large quantities of sICAM-1. However, following stimulation with IFNγ, high levels of sICAM-1 were detected in tissue culture supernatants (Figure 34). Following 24 hours of stimulation, low levels of sICAM-1 were produced by each line. The maximal levels of sICAM-1 were not detected until after 48 (MGH-U1), 72 (RT4 and SD) and 96 hours (RT112). Maximal levels in excess of 200ng per 10^6 cells were produced by the SD cell line. Assuming a molecular weight of 95kDa, this corresponds to the secretion of approximately $1.3x10^6$ molecules per cell during a 72 hour period from the initiation of stimulation with IFNγ, as opposed to only $6x10^3$ molecules per cell without stimulation.

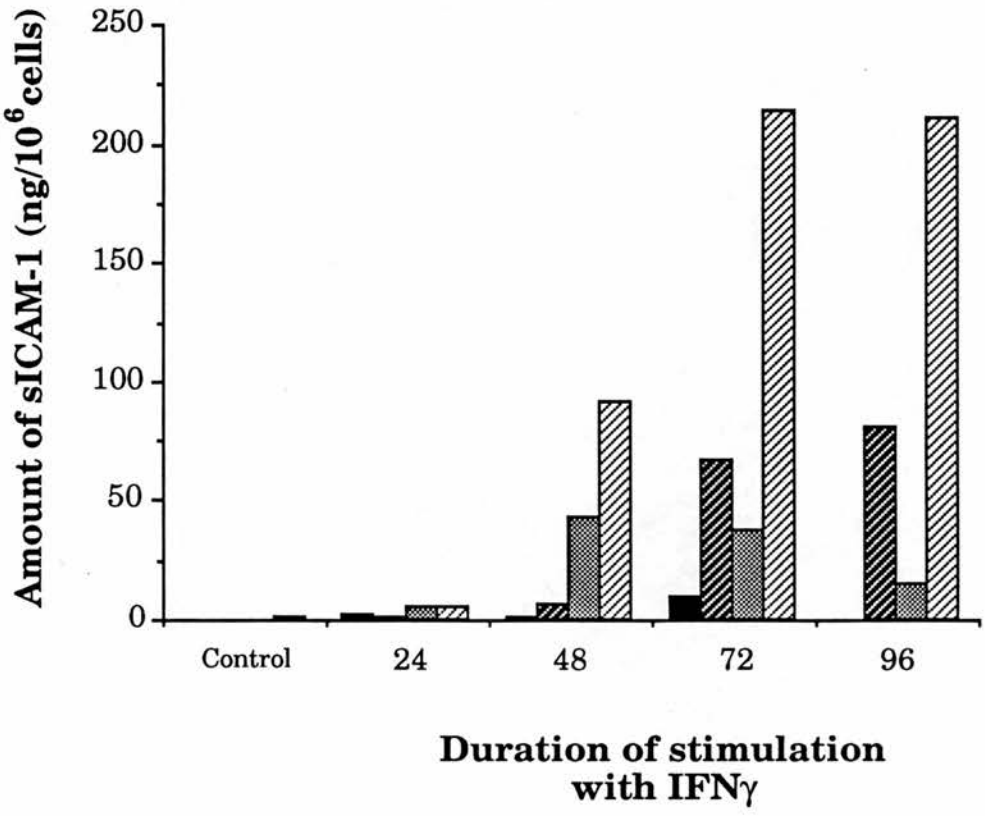
3.2.16 Supernatants from LAK cell cultures induce the expression of ICAM-1 on bladder cancer cells

As mentioned in *3.1.11* several cytokines are produced by PBMC following stimulation with IL-2. The effect of such cytokines on ICAM-1 expression was studied. It had previously been demonstrated that IL-2 does not modulate ICAM-1 expression, therefore any effects were likely to be due to secondary cytokines.

Following 24 hours treatment with a 1:2 dilution of 6 day LAK cell supernatant, ICAM-1 expression was induced on RT112 (51%±3) and MGH-U1 (90%±4). Pretreatment with polyclonal antibodies to IFNγ (80% reduction in effect), but not TNFα (8% reduction), almost completely negated the effect of LAK supernatant. Simultaneous treatment with

both antibodies did not decrease the effect any further than with anti-IFNγ alone. Whether such cytokines produced by tumour infiltrating cells would have similar effects on tumour cells remains to be investigated. It is interesting than despite cytokine neutralization, ICAM-1 levels did not return to constitutive levels, indicating the presence of a further inducer of ICAM-1 (such as IL-1).

Figure 34. The secretion of soluble ICAM-1 by bladder cancer cells



The production of soluble ICAM-1 (sICAM-1) by four bladder cancer cell lines following stimulation with 100U/ml of recombinant IFNγ for the indicated periods. Control is the levels of production in the absence of IFNγ. The four cell lines used were RT4 (■), RT112 (▨), MGH-U1 (▩), and SD (▨). The production of sICAM-1 was detected using an ELISA kit and is expressed as ng per million cells.

3.3 Functional evaluation of the role of adhesion molecules in LAK mediated cytotoxicity against bladder cancer cells

The work so far has shown that bladder cancer cells are differentially susceptible to killing mediated by IL-2 activated LAK cells. Scanning electron micrography has demonstrated an intimate relationship between the LAK cell and bladder cancer cells. The differential sensitivity of the target cells would appear to correlate with the expression of one particular adhesion molecule, ICAM-1, expressed by the target cell. Using flow cytometry, the existence of the ligand for ICAM-1 has been confirmed on the potential effector cells. However, several points have been raised. Firstly, a significant percentage of killing would appear to exist in the absence of ICAM-1 and ICAM-2. Secondly, unactivated effector cells express high levels of LFA-1 yet are unable to kill bladder cancer cells. However, NK sensitive targets are killed, thus indicating the existence of a differential mechanism of killing. Thirdly, ICAM-1 is sensitively regulated by a variety of cytokines, most of which lead to the increased expression of this molecule. The following series of experiments were designed to further dissect the role of various adhesion molecules in the intimate relationship between effector and target cell.

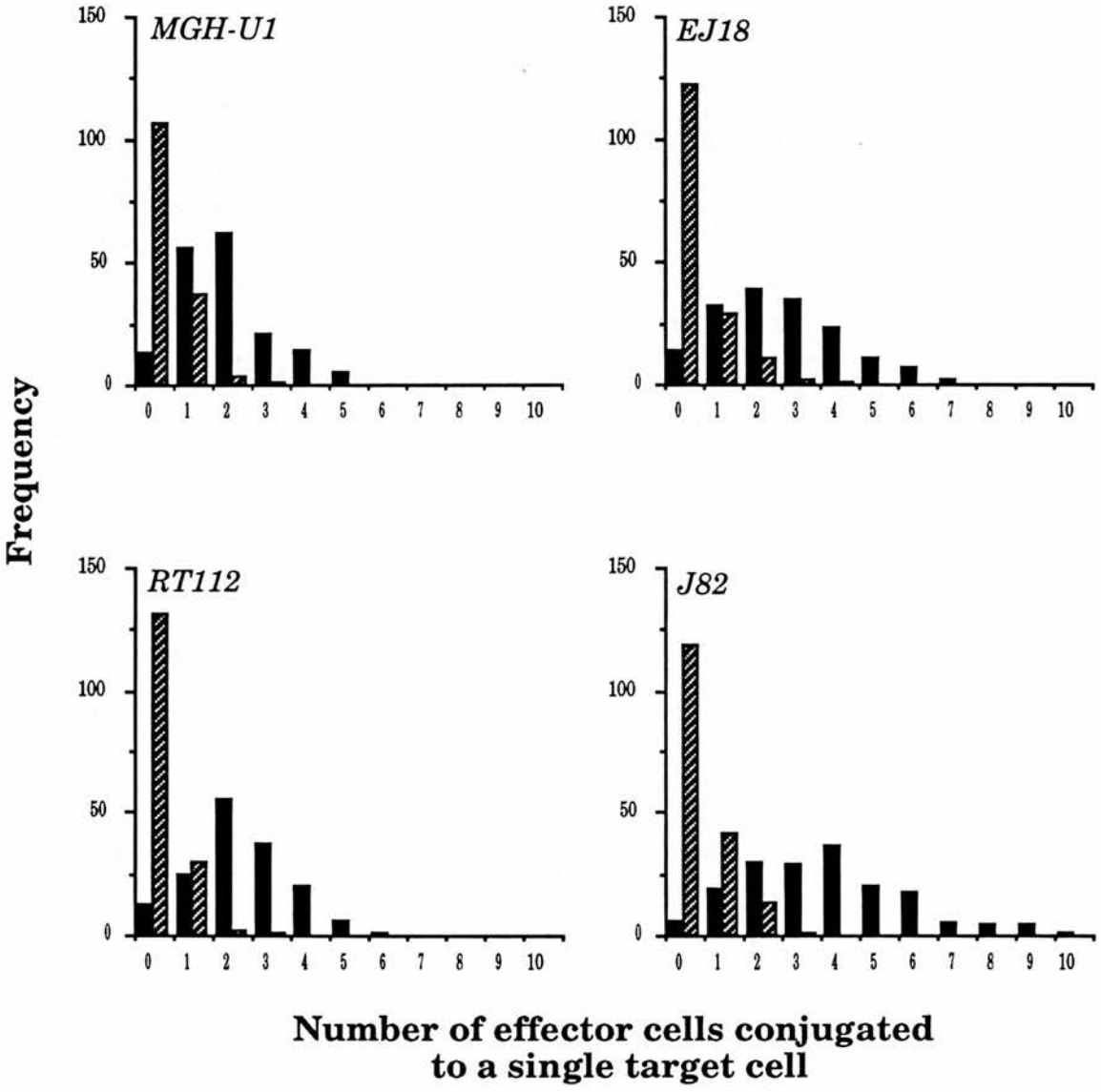
3.3.1 Immunohistochemical detection of adhesion molecules in conjugates between effector and target cells

Immunohistochemistry techniques provide us with a powerful method for the visualization of antigens. When applied to cell biology, they can reveal minute differences in the distribution of antigen, whether surface bound or cytoplasmic, equally distributed on the surface of the cell

or localized towards one pole. Immunohistochemistry for cell surface antigens (when carefully undertaken) provides additional information to that give by flow cytometry. The aim of this series of experiments was to identify molecules expressed by target and effector cells and to visualize conjugation events between the two cell types. Conjugates were formed and using cytospin were transferred to a glass slide. Following fixation the cells were stained for the expression of various adhesion molecules using monoclonal antibodies. The frequency of the number of effector cells conjugated to individual target cells is shown in Figure 35.

Cytospins of target and effector cells (10:1) were stained for ICAM-1 and the number of conjugates per target cell was counted; the data is shown in table 6. There was a significantly greater number of effector cells conjugated to a single ICAM-1 expressing target cell than an ICAM-1 negative target cell for all four cell lines tested ($p<0.02$). Differences were also evident between the individual cell lines in the case of ICAM-1 expressing target cells, however, these differences failed to reach significance ($p>0.2$). When the data was corrected for the percentage of cells expressing ICAM-1, differences were evident between the cell lines. A greater number of effector cells was seen to conjugate to the cell lines EJ18 and J82 than to MGH-U1 and RT112 ($p<0.02$). No difference was detected between RT112 and MGH-U1. The correlation between the susceptibility of targets to LAK activity and the number of LAK cells conjugated to each target cell is shown in Figure 36. Although only four data points exist, the correlation is significant to at least the 1% level.

Figure 35. The number of LAK cells binding per target cell



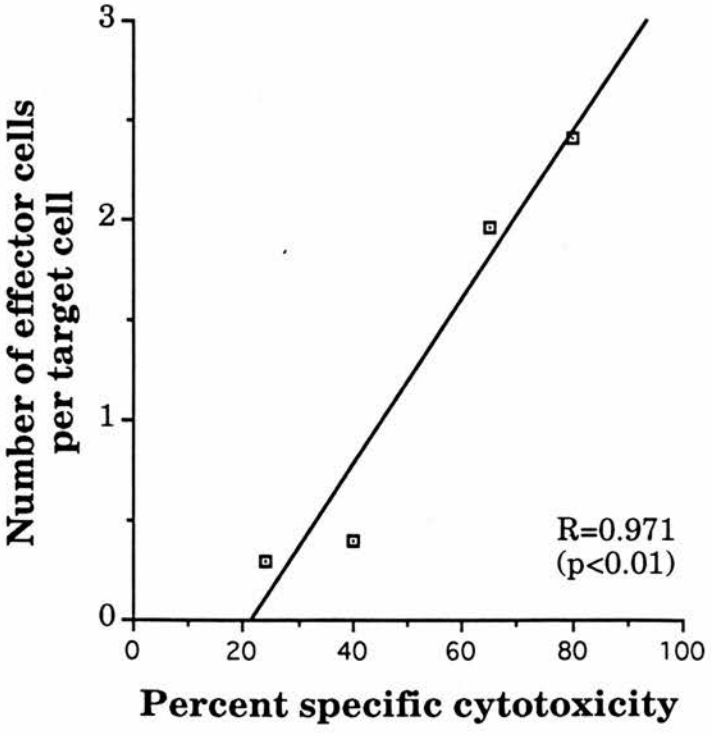
The frequency of the number of effector cells conjugated to a single target cell is shown. Targets and effector cells were mixed at a 1:10 ratio and briefly centrifuged prior to preparing cytospins. Following immunohistochemical staining for ICAM-1, the number of effector cells conjugated to either ICAM-1 positive (■), or ICAM-1 negative (▨) cells was counted. The results shown are for four cell lines as indicated.

Table 6. Summary of the immunohistochemical binding data for conjugates formed between LAK and TCC cells.

Cell Line	Number of effector cells per target ICAM-1 +	ICAM-1 -	Number of effector cells per target
MGH-U1	1.9±1.2	0.3±0.5	0.40±0.6
EJ18	2.6±1.6	0.3±0.5	1.96±1.2
RT112	2.3±1.3	0.2±0.4	0.3±0.4
J82	3.7±2.0	0.3±0.5	2.41±1.4

The data presented is based on information provided in figure 35. The results show the mean number of effector cells (±1sd) for four target cell lines. This number is calculated from the number of effector cells per ICAM-1 positive and negative target cell and is adjusted for the mean percentage of cells expressing ICAM-1.

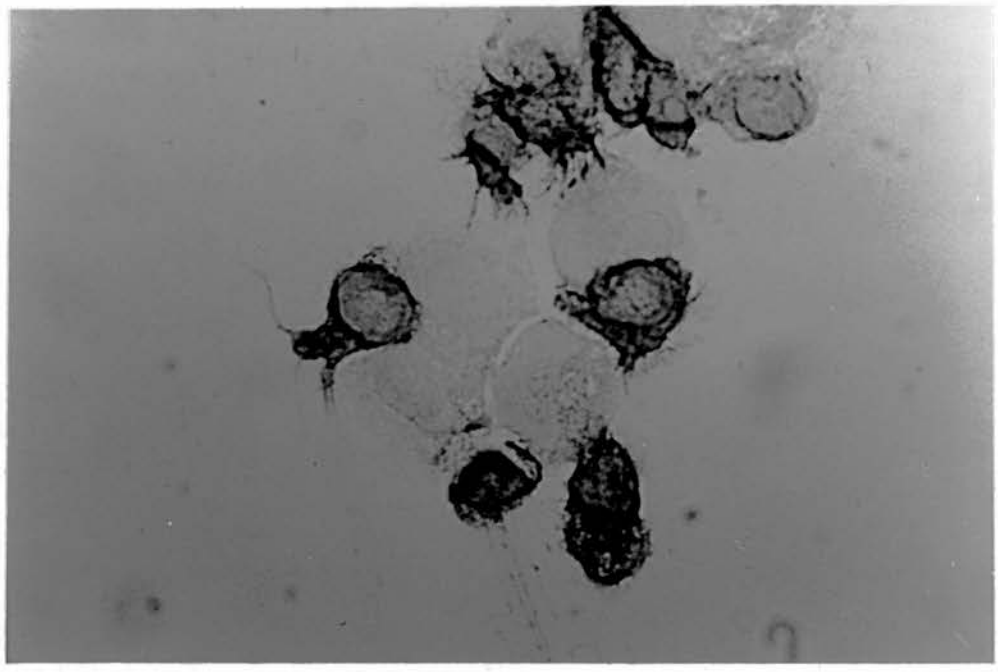
Figure 36. Correlation between conjugation and LAK activity

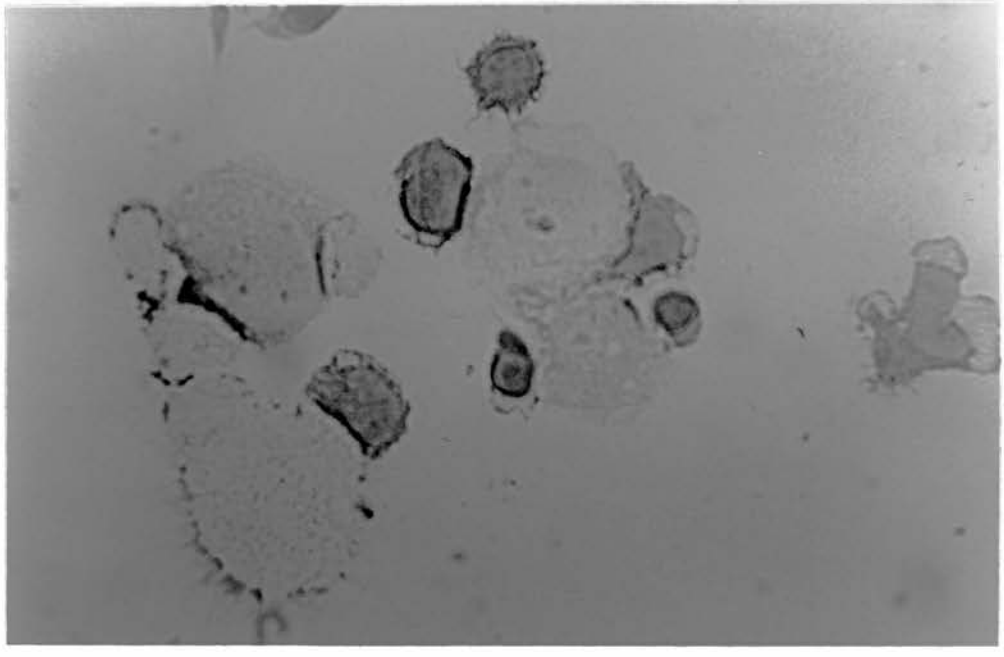


Shown is the correlation between susceptibility to LAK killing and the number of LAK cells per target cell for four target cell lines. Also shown is the correlation coefficient (R) and the probability (p).

When conjugate cytospins were stained using a primary monoclonal antibody to LFA-1, it was noted that all effector cells which formed conjugates with target cells, expressed this antigen. Furthermore, both lymphocytes and LGL were observed to form intimate conjugates with bladder cancer cells. Representative results of the immunohistochemistry are shown in plates 4 and 5.

Plates 4 and 5.





Immunohistochemical staining for CD11a of conjugates formed between IL-2 activated LAK cells and RT4 (upper) or MGH-U1 (lower).

3.3.2 The effect of adhesion blockade of ICAM-1 and ICAM-2 on target cells

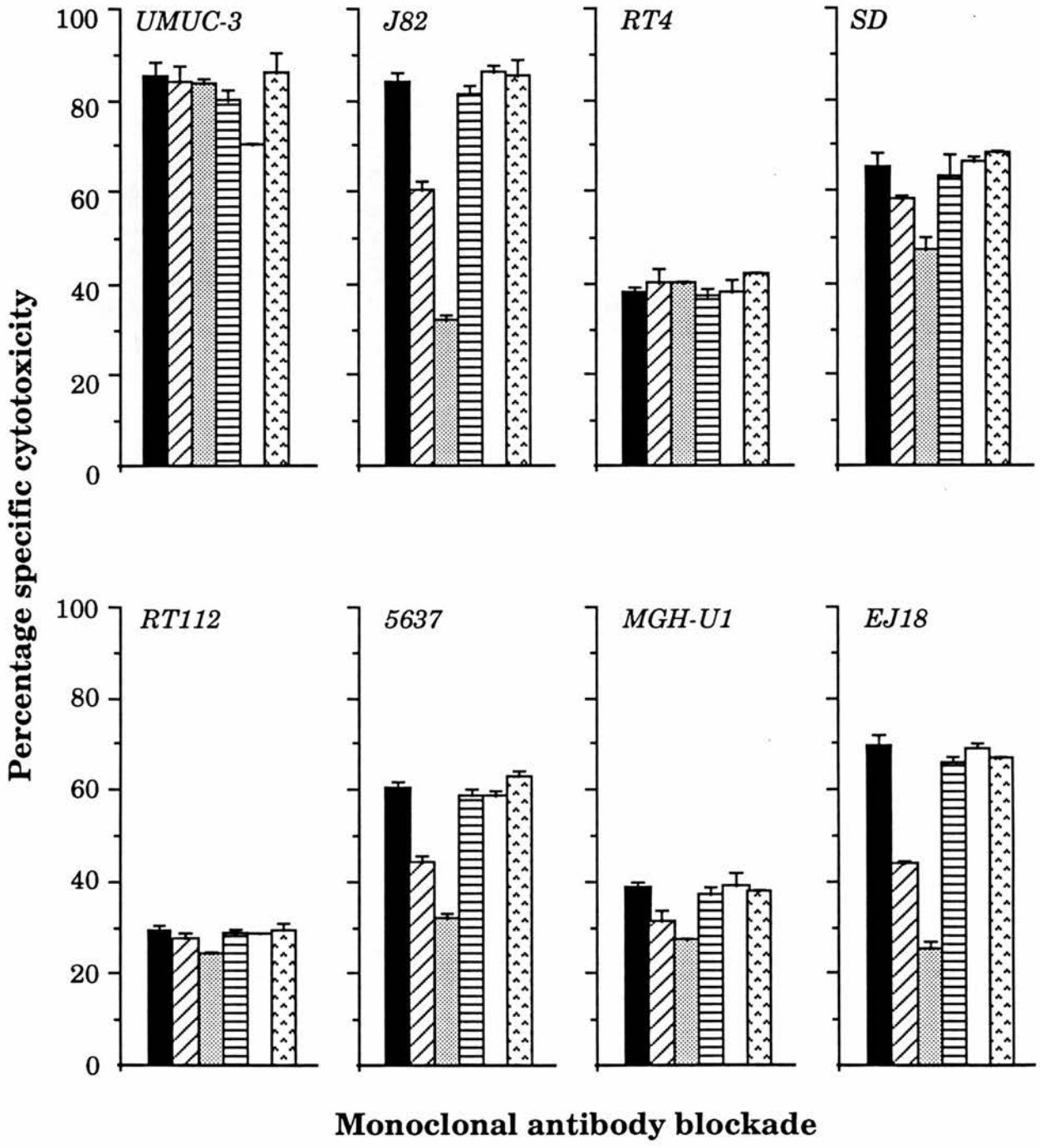
In order to demonstrate the role of ICAM-1 and ICAM-2 as ligands for effector cells, targets were pretreated with monoclonal antibodies known to inhibit the function of various adhesion molecules.

When targets which were known to strongly constitutively express ICAM-1 (J82, 5637, EJ18, SD) were pretreated with blocking antibodies to ICAM-1, a significant decrease in their susceptibility to LAK mediated cytolysis was observed (Figure 37). Furthermore, when antibodies were also present for the duration of the cytotoxicity assay a further decrease in specific cytotoxicity was observed. The mean maximal suppression of specific cytotoxicity was as follows: J82-70%, 5637-47%, EJ18-62%, and SD-28%. When the two cell lines which constitutively express ICAM-2 were pretreated with anti ICAM-2 no significant decrease in specific cytotoxicity was observed ($p>0.2$). However, when these antibodies were present throughout the assay period a decrease was observed in the killing of UMUC-3, 17%. Only a minor decrease was observed in the killing of the G2 cell line RT112, however, this did not reach significance. Antibodies to either ICAM-1 or ICAM-2 failed to reduce the specific cytotoxicity achieved against RT4 (G1).

The presence of antibodies to adhesion molecules which were not expressed by the target cell did not affect the sensitivity of that target to LAK cells. Furthermore, the presence of antibodies to HLA class II molecules did not inhibit specific cytotoxicity for any of the eight cell lines.

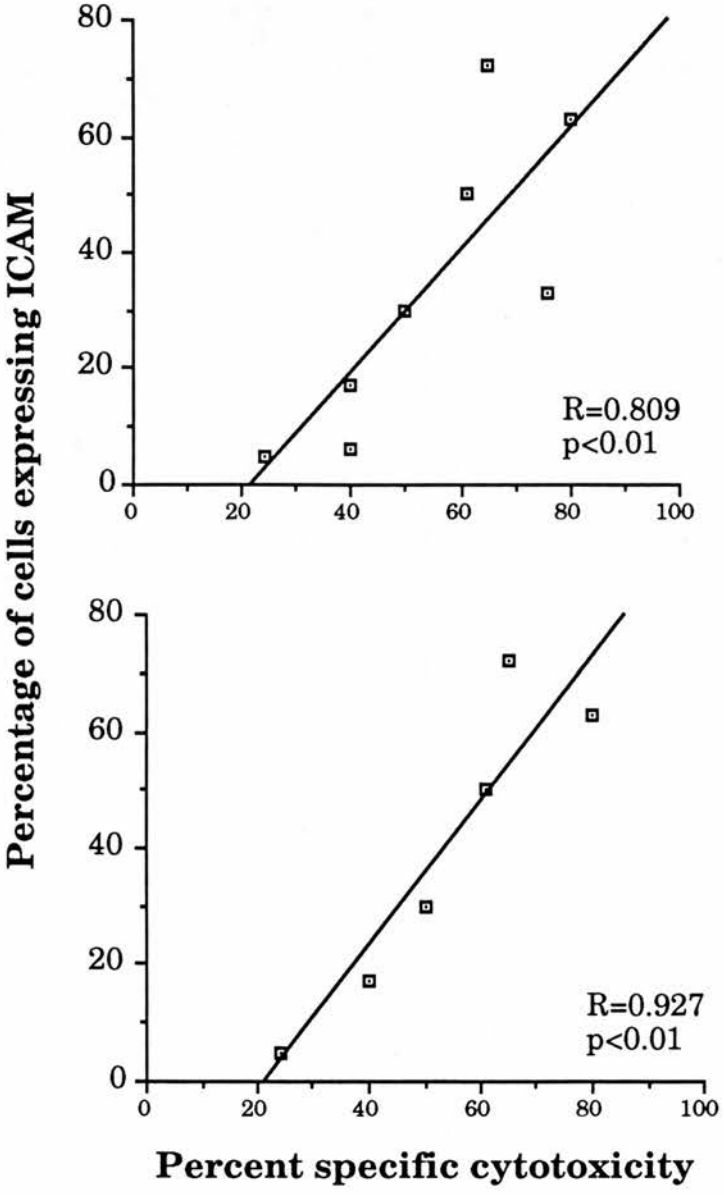
When the percentage of target cells which expressed ICAM-1 (and or ICAM-2) was plotted against the specific cytotoxicity obtained against such cells a significant correlation was observed (Figure 38). With only

Figure 37. Adhesion blockade of target cells



The functional dissection of the role of adhesion molecules in the LAK cell mediated cytotoxicity against bladder cancer cell lines. A standard 4 hour chromium release assay was performed at an effector to target ratio of 40:1. Prior to assay, the target cells were incubated with a variety of monoclonal antibodies for 30 minutes at a concentration of 10μg/ml (pre) or antibodies were present throughout the assay period (during). The monoclonal antibodies employed were as follows; none (■), anti-ICAM-1 pre (▨), anti-ICAM-1 during (▩), anti ICAM-2 pre (▤), anti-ICAM-2 during (□), anti-HLA class II pre and during (▣).

Figure 38. The correlation between constitutive ICAM expression and susceptibility to LAK mediated killing



In the upper figure the correlation is shown for the expression of both ICAM-1 and ICAM-2 with LAK susceptibility. The lower figure shows the correlation with ICAM-1 alone. Also shown is the correlation coeficient (R) for both regressions.

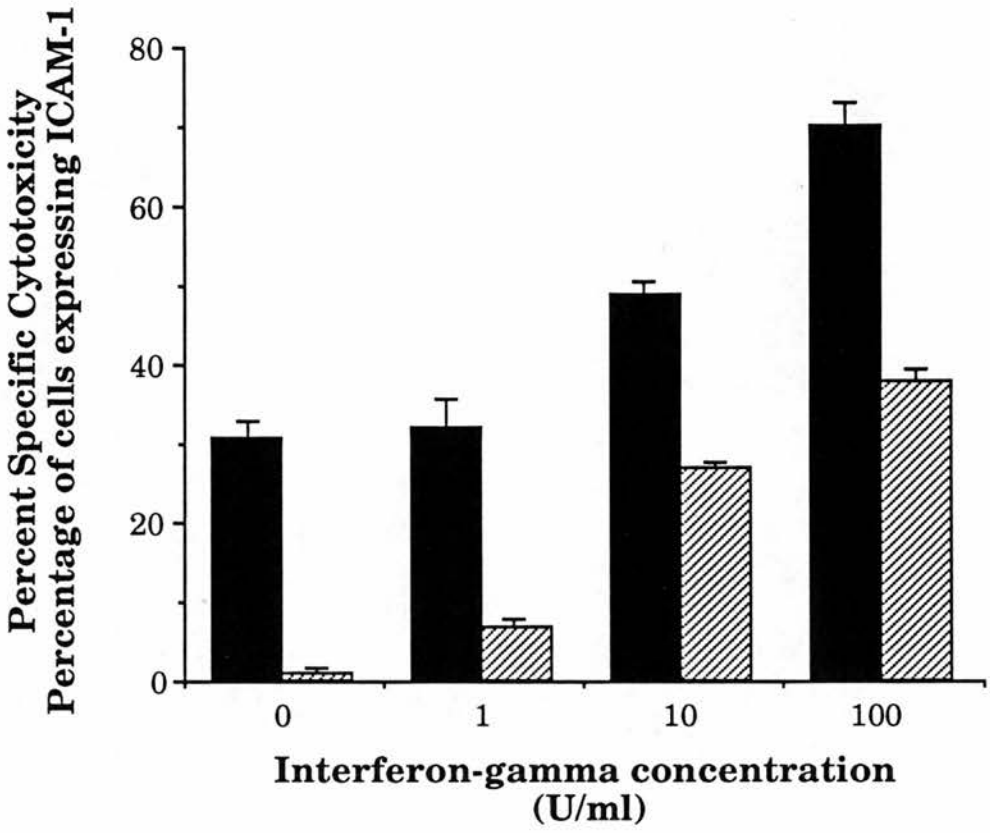
two cell lines expressing ICAM-1 it was not possible to assign any significance to their correlation. However, it is possible that the relationship to LAK activity is different for ICAM-1 and ICAM-2.

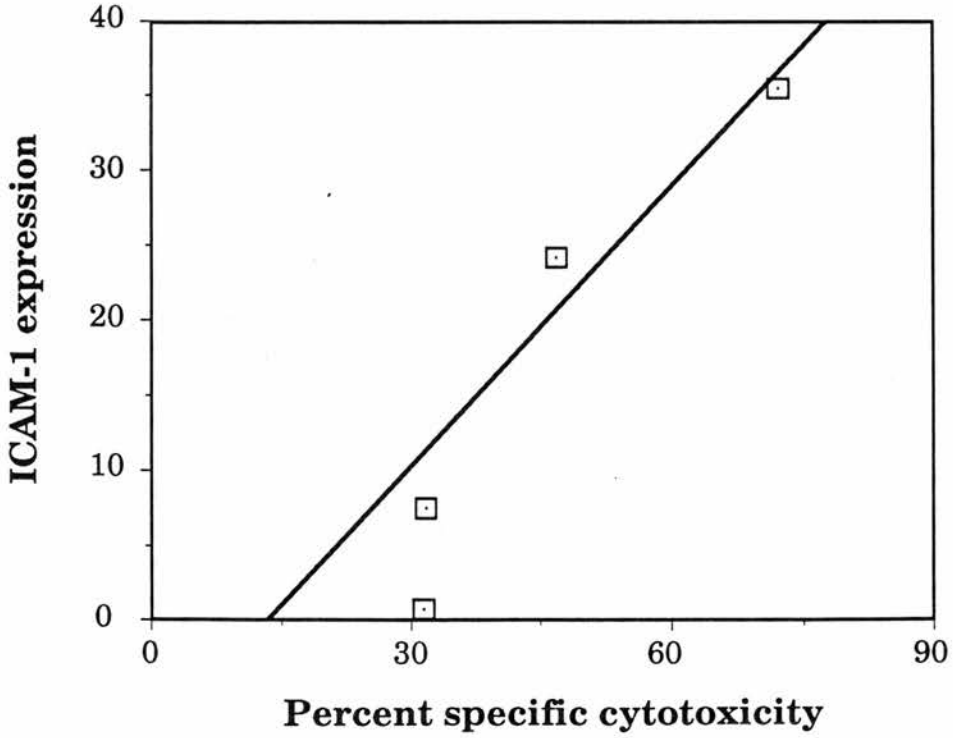
3.3.3 Interferon-gamma treatment of tumour cells increases their susceptibility to LAK mediated cytolysis

If the cell adhesion molecule ICAM-1 serves as a ligand for the LAK effector cell then, in a non-linear manner, a cell with increased ICAM-1 expression on its surface should have an increased susceptibility to LAK mediated killing. In order to test this hypothesis tumour cells were first incubated for 24 hours with IFNγ at a maximal concentration of $100Uml^{-1}$. The specific cytotoxicity achieved against these targets was then compared to that against untreated target cells. The percentage of cells expressing ICAM-1 was also measured.

Stimulation of the RT4 (G1) cell line with increasing concentrations of IFNγ resulted in an apparent increase in the susceptibility of the tumour cells to cytolysis by LAK effector cells (Figure 39). Although incubation with $1Uml^{-1}$ of IFNγ resulted in a small increase in the percentage of cells expressing ICAM-1, there was no corresponding increase in LAK activity. Stimulation of the tumour cells with higher concentrations of IFNγ did produce an increase in killing. Incubation with $10Uml^{-1}$ gave an increase in killing of 35% of the control value for untreated cells. Stimulation with $100Uml^{-1}$ resulted in an increase in specific killing of 215%. The correlation line between increased ICAM-1 expression and increased sensitivity to LAK cells is shown (Figure 39). The fit to this line is good for all points bar that obtained with unstimulated cells (the correlation coefficient gives $p<0.01$).

Figure 39. Interferon-gamma increases target suceptibilty



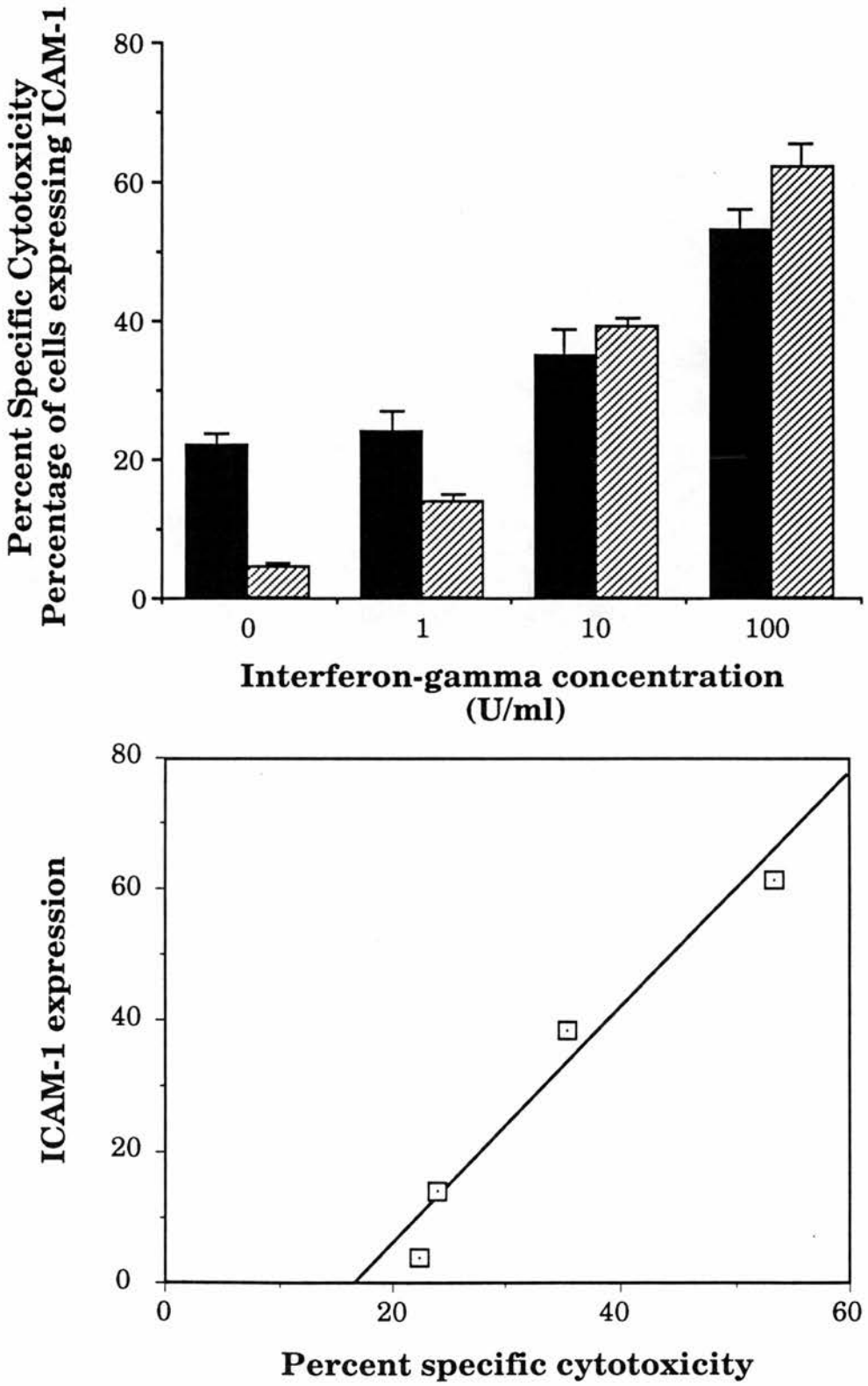


The relationship between IFNγ induced ICAM-1 expression by RT4 and increased sensitivity to LAK mediated cytotoxicity. Cells were incubated for 24 hours with the indicated dose of IFNγ following which both the specific cytotoxicity (■) and the percentage of cells expressing ICAM-1 (▨) were determined (upper figure). The correlation between these two parameters is shown in the lower figure.

When the RT112 cell line was incubated with increasing concentrations of IFNγ an increase in the susceptibility of the cells to LAK mediated killing was also observed (Figure 40). The increased susceptibility was not evident after stimulation with $1Uml^{-1}$ despite an increase in the expression of ICAM-1. However, following stimulation with $10Uml^{-1}$ a 40% increase in specific cytotoxicity was noted. The augmentation of specific cytotoxicity was even greater when cells were treated with $100Uml^{-1}$ IFNγ. A 250% increase in LAK activity was observed over the control value for unstimulated cells. A corresponding increase in ICAM-1 expression was also observed. When the percentage specific cytotoxicity and the ICAM-1 expression following stimulation with increasing doses of IFNγ were plotted against each other a significant correlation was found ($p<0.02$) (Figure 40).

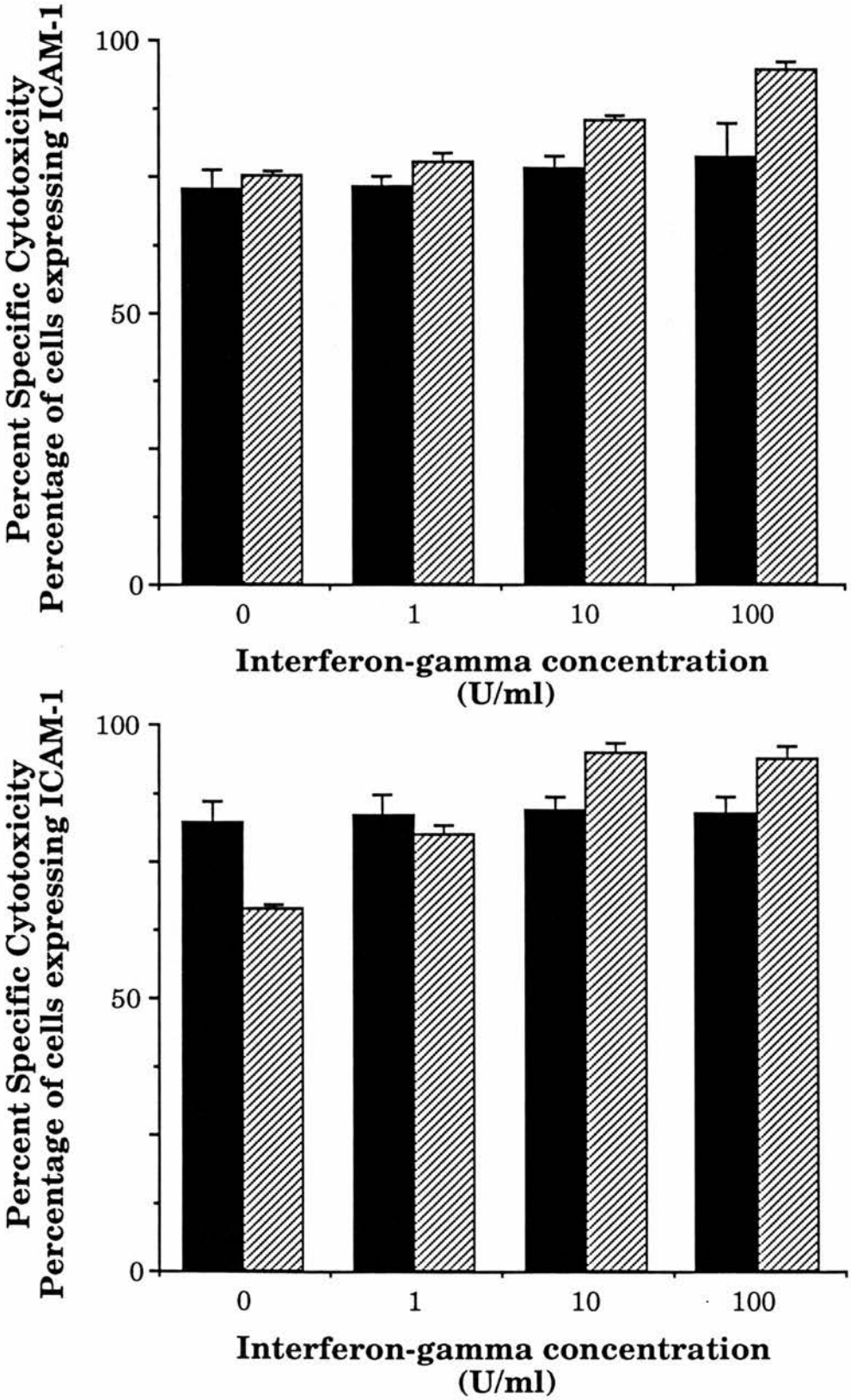
Stimulation of the high grade cell lines EJ18 and J82 with $100Uml^{-1}$ of IFNγ was found to increase their expression of ICAM-1. However, no significant increase in specific cytotoxicity against these targets was observed (Figure 41). Previous data has shown that the TCC cell line UMUC-3 (G3) cannot be stimulated to express ICAM-1, even when exposed to high concentrations ($>1000Uml^{-1}$) and prolonged periods of exposure (>200 hours). When this cell line was exposed to IFNγ and its susceptibility to LAK killing subsequently determined, there was no evidence of an increase in vulnerability to LAK cells (Figure 42).

Figure 40. Interferon-gamma increases target suceptibilty



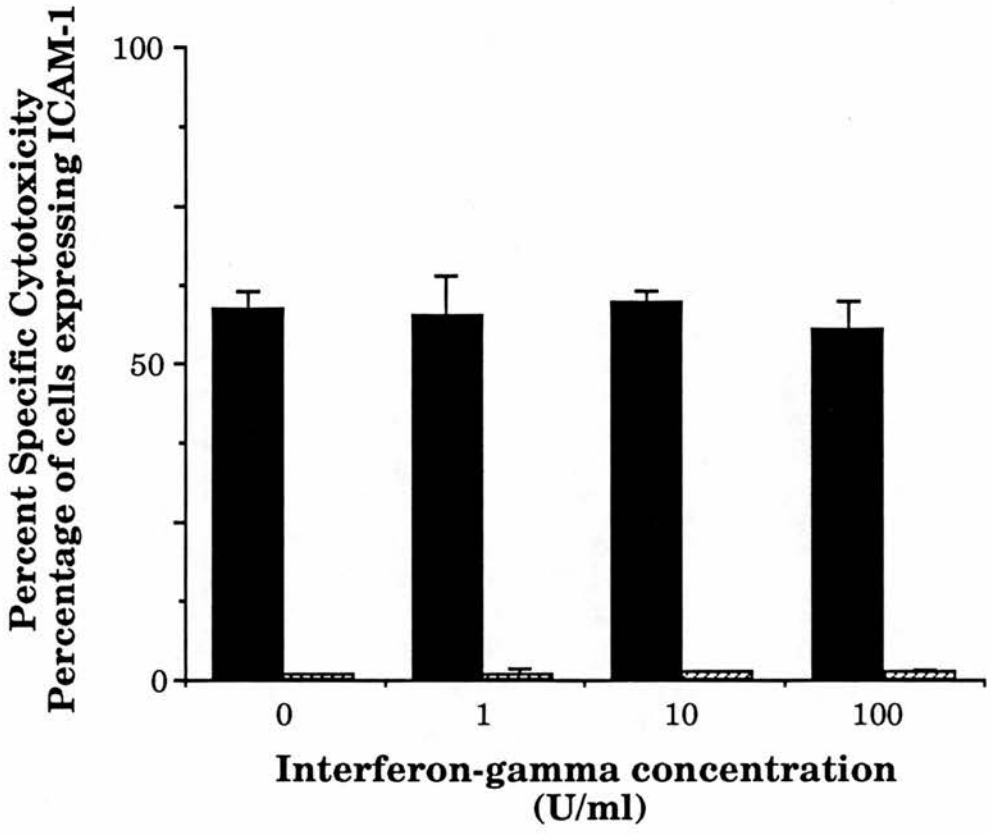
The relationship between IFNγ induced ICAM-1 expression by RT112 and increased sensitivity to LAK mediated cytotoxicity. Cells were incubated for 24 hours with the indicated dose of IFNγ following which both the specific cytotoxicity (■) and the percentage of cells expressing ICAM-1 (▨) were determined (upper figure). The correlation between these two parameters is shown in the lower figure.

Figure 41. Interferon-gamma fails to increase target suceptibilty



The relationship between IFNγ induced ICAM-1 expression by EJ18 and J82 and sensitivity to LAK mediated cytotoxicity. Cells were incubated for 24 hours with the indicated dose of IFNγ following which both the specific cytotoxicity (■) and the percentage of cells expressing ICAM-1 (▨) were determined. Upper figure gives the results for EJ18 and the lower figure for J82. Bars represent means of triplicates and error bars indicate 1 standard deviation.

Figure 42. Interferon-gamma fails to increase target suceptibility



The relationship between IFNγ induced ICAM-1 expression by UMUC-3 and its sensitivity to LAK mediated cytotoxicity. Cells were incubated for 24 hours with the indicated dose of IFNγ following which both the specific cytotoxicity (■) and the percentage of cells expressing ICAM-1 (▨) were determined. Bars represent means c triplicates and error bars indicate 1 standard deviation.

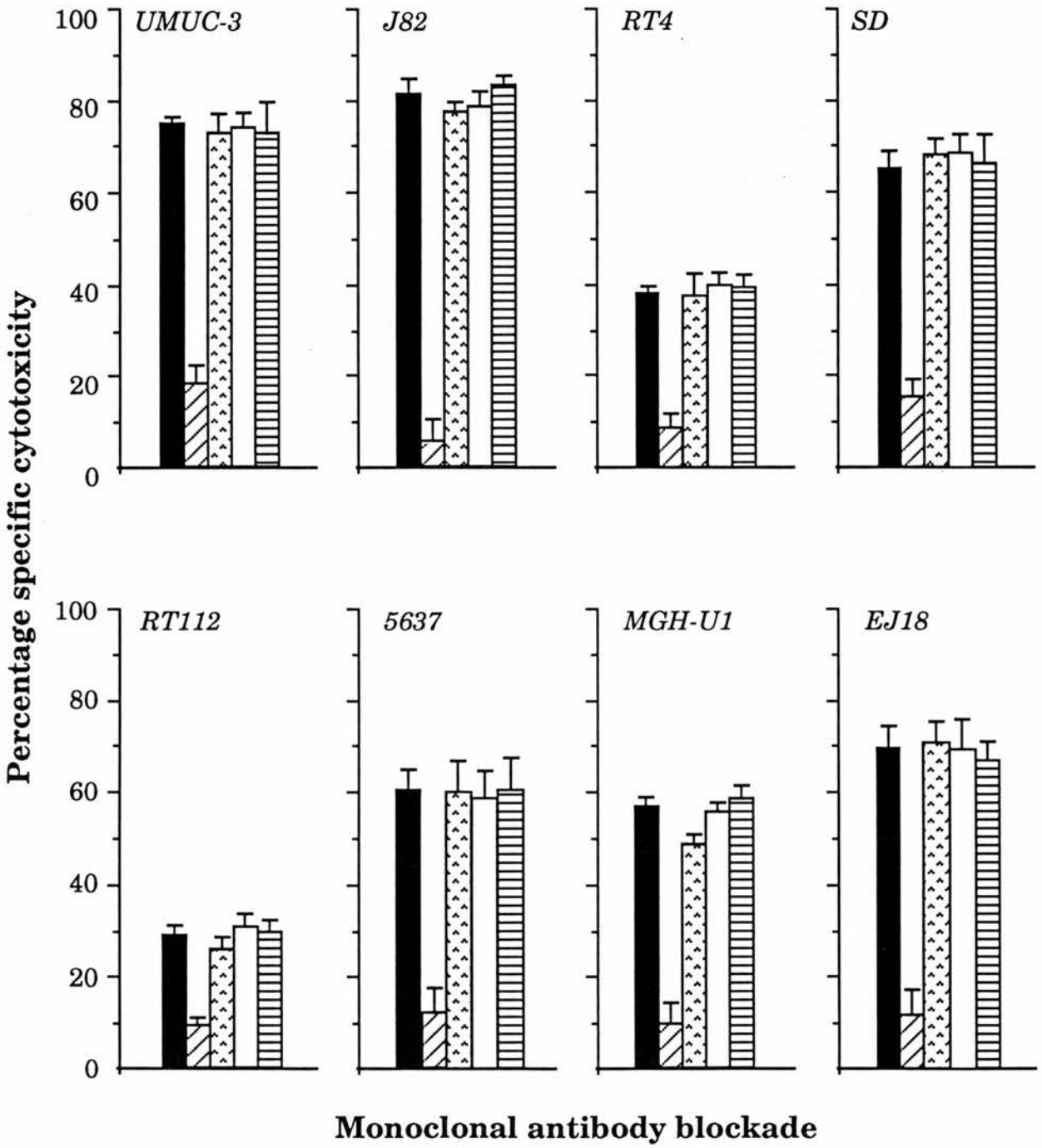
3.3.4 Adhesion blockade studies concerning the effector cell

The data presented so far concerning the function of adhesion molecules in the activity of LAK cells against bladder cancer targets has concentrated mainly upon the function of adhesion molecules expressed by the target cell. Using various monoclonal antibodies these molecules have been shown to partake in the LAK response. If this is not artifactual then similar adhesion blockade studies with respect to the ligands on effector cells should realize similar findings. It was on this premise that investigations into the counter-receptor for ICAM, namely LFA-1, were undertaken. Using monoclonal antibodies known to inhibit the function of various adhesion molecules the role of such structures as expressed by the effector cell was investigated.

When effector cells were pretreated with monoclonal antibodies to ICAM-1 or ICAM-2 no changes in the specific cytotoxicity exhibited against any of the targets was observed (Figure 43). Antibodies to LFA-2 gave similar results on most of the cell lines tested. However, LAK cells which had been pretreated with anti-LFA-2 antibodies and then used to kill the line MGH-U1 had a slight but significantly decreased ability to do so ($p<0.02$). A depression of 14% of the control value was consistently observed.

The pretreatment of LAK cells with monoclonal antibodies to LFA-1 alpha chain (CD11a) resulted in a marked and significant decrease in the ability of LAK cells to kill their bladder cancer targets. The percentage decrease ranged from 70% (RT112) to 96% (J82) with a mean depression of 83% of control value. Intermediate levels of depression in activity were achieved against other cell lines; 76% (UMUC-3), 84% (MGH-U1), 77% (RT4), 75% (SD), 80% (5637), and 83% (EJ18).

Figure 43. Anti-LFA-1 antibodies inhibit LAK cytotoxicity



The functional dissection of the role of adhesion molecules in the LAK cell mediated cytotoxicity against bladder cancer cell lines. A standard 4 hour chromium release assay was performed at an effector to target ratio of 40:1. Prior to assay, the effector cells were incubated with a variety of monoclonal antibodies for 30 minutes at a concentration of 10μg/ml. The monoclonal antibodies employed were as follows; none (■), anti-LFA-1 (▨), anti-LFA-2 (▣), anti-ICAM-1 (□), anti-ICAM-2 (⊟). Bars show the mean of triplicate determinations, error bars indicate the level of 1 standard deviation.

When the percentage depression in LAK activity for target cell blockade and effector cells blockade are compared, either per individual cell line or as the mean of all eight cell lines, a marked difference is observed. The mean level for target blockade is 31±24% as compared to 82±9.7% following effector cell blockade for all eight cell lines. Therefore, as there is a portion of LAK activity which is LFA-1 dependent but independent of the function of both ICAM-1 and ICAM-2, the existence of a further ligand for LFA-1 on bladder cancer cell lines is suggested.

3.3.5 The formation of stable conjugates between effector and target cells

As various adhesion molecules appear to be involved in the activity of LAK cells against bladder cancer targets, it is reasonable to assume that effector and target cells form stable conjugates in order for cytotoxic events to be initiated. Using a modified flow cytometric-based conjugation assay (Caverec *et al* 1990) the rate of conjugation for each of the bladder cancer cell lines was studied. An effector to target ratio of 10:1 was chosen as a compromize between the cytotoxicity assay and the constraints of flow-rate. Conjugation was carried out in complete medium at 37°C, these conditions being chosen for their similarity to previous experiments concerning cytotoxicity.

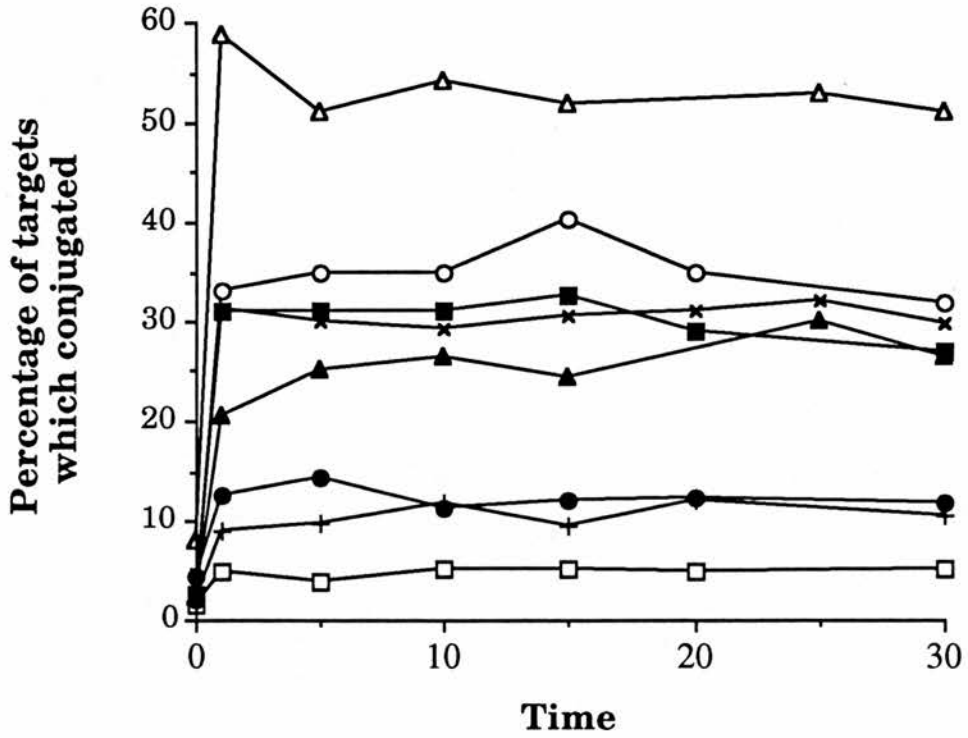
The percentage of targets which were conjugated to at least one effector cell was expressed as a function of time. Assay before pelletting the cells gave t=0. At this time point the number of conjugates was extremely low, usually less than 1%. Immediately following pelletting and vigorous resuspension, t=1, the conjugates were again assayed. A representative time course for each cell line is shown in Figure 44. Following centrifugation, conjugation was observed to be a rapid (<1

minute) and stable event. In fact, the maximal level of constitutive conjugation was observed at 1 minute. When cells were subjected to vigorous vortexing or repeated pipetting, no decrease in the percentage of conjugates was observed (data not shown). Although previously a significant correlation had been demonstrated between ICAM-1 expression and LAK activity, interestingly there was no such significant correlation ($p>0.2$) between the constitutive susceptibility to LAK mediated cytotoxicity and the ability to form conjugates (Figure 45). The mean results from repeated conjugation experiments are shown in table 7. The two low grade cell lines RT4 and UMUC-3 were found to conjugate the least, G2 and G3 cell lines forming more stable conjugates. In apparent contradiction to the cytotoxicity assay, RT112 was found to form the most conjugates, suggesting further mechanisms other than conjugation are involved in LAK mediated cytolysis.

3.3.6 Fresh PBMC do not form conjugates with bladder cancer cells

In order to more fully understand the mechanisms of LAK activity against bladder cancer cell lines, the ability of fresh PBMC to conjugate with target cells was investigated. As the expression of LFA-1 by PBMC is high, the question of ligand activation could be addressed. Fresh PBMC were unable to conjugate with any of the bladder cancer cell lines investigated (data not shown). The level of conjugation was never greater than the control levels obtained without centrifugation.

Figure 44. The kinetics of conjugate formation

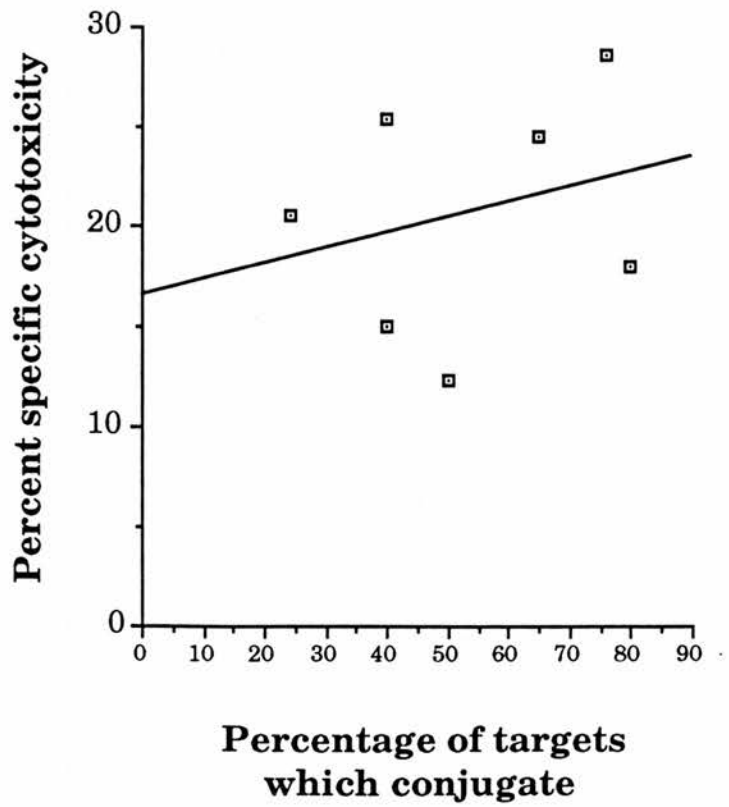


A representative time course of conjugate formation between LAK cells and eight bladder cancer cell lines. Target cells were labelled with dihydroethidium and effector cells with CFDA.AM. The fluorescence was determined using a flow cytometer. Conjugation was initiated by briefly centrifuging the cells in complete medium at 37℃, followed by rapid resuspension. The target cells used were as follows; RT4 (+), RT112 (Δ), MGH-U1 (■), EJ18 (○), J82 (▲), 5637 (□), UMUC-3 (●), and SD (×).

Table 7. The mean level of conjugation as determined by flow-cytometry

Cell line	Percent specific cytotoxicity	Percent targets which conjugate
RT4	40	15.0±4.0
RT112	76	28.6±8.0
MGH-U1	24	20.5±7.0
EJ18	40	25.4±6.7
J82	65	24.5±2.9
SD	80	18.0±4.9
UMUC-3	50	12.3±3.0

Figure 45. Lack of correlation between LAK activity and ability to conjugate



The correlation between constitutive susceptibility to LAK mediated cytotoxicity and conjugate formation. The correlation coefficient does not reach significance ($p>0.2$).

3.3.7 Antibody blockade of conjugate formation

The studies have demonstrated a role for the LFA-1/ICAM interaction in the activity of LAK cells against bladder cancer cells. In the presence of monoclonal antibodies to LFA-1 and ICAM-1, the specific cytotoxicity of LAK cells was reduced. In order to further illustrate the role of these adhesion molecules and to dissect the mechanisms involved in LAK activity, adhesion blockade was undertaken with the conjugate assay.

When effector cells were pretreated with monoclonal antibodies to CD11a, their ability to form conjugates with bladder cancer target cells was negated (Figure 46). The mean level of conjugation in the presence of antibodies to CD11a was reduced by 70.5%±23. The level of conjugation of UMUC-3 was not greatly reduced by anti-LFA-1 antibodies (20% reduction). When the cells lines known to express moderate or high levels of ICAM-1 (MGH-U1, EJ18, SD) were incubated with anti-ICAM-1 antibodies and marked reduction in conjugation was observed. This level of reduction was equivalent to that achieved by antibodies to LFA-1. The level of conjugation of cell lines RT4 and RT112 was not greatly reduced by antibodies to either ICAM-1 or ICAM-2. Finally, when UMUC-3 was pre-incubated with antibodies to ICAM-1 and ICAM-2 a reduction in the level of conjugation was observed. In the case of anti-ICAM-2 this level of reduction was greater than that achieved with anti-LFA-1.

3.3.8 Conjugation to target cells is an energy dependent event

The requirement for an active process in conjugation was investigated by using sodium azide to inhibit metabolic processes. When LAK cells were treated with sodium azide the level of conjugation was

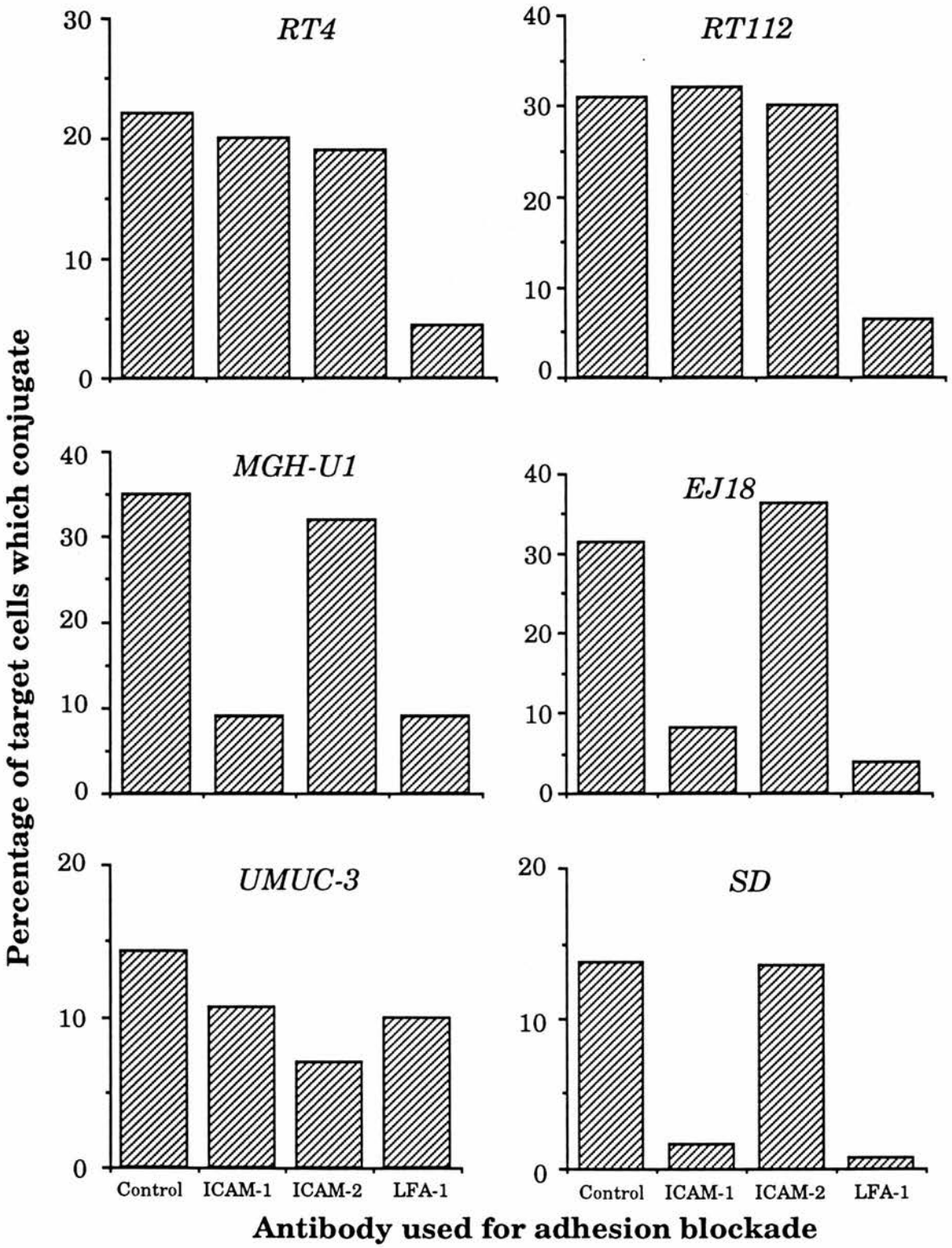
usually less than 3% (Figure 47). However, similar treatment of the target cells did not affect their ability to form conjugates (data not shown). When both cell populations were separately incubated with sodium azide prior to conjugate formation, no decrease below that obtained with effector cells alone was observed.

3.3.9 The conjugation of effector and target cells is dependent upon divalent cations

Previous work by Marlin and Dransfield has elegantly demonstrated the role of several divalent cations, notably magnesium, manganese and calcium, in the function of LFA-1 (Dransfield *et al* 1989. Marlin *et al* 1987). The role of such cations in the function of LFA-1 as a counter-receptor for ligands on bladder cancer cells was investigated with the aid of the above conjugate assay.

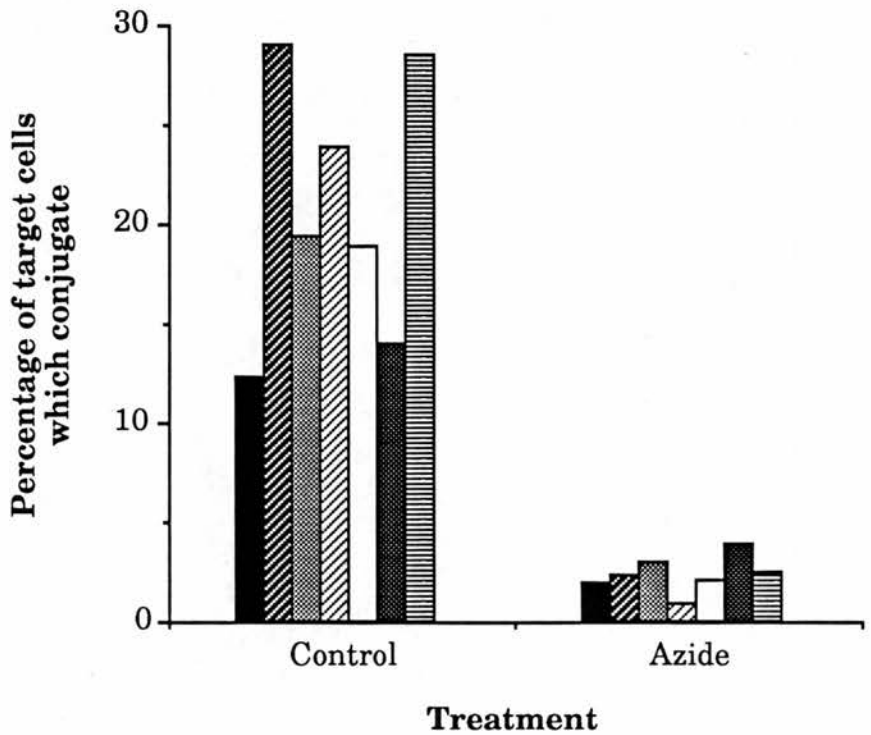
When calcium ions were removed from complete medium with the aid of 1mM EGTA, a small but consistent reduction in the level of conjugation was noted (Figure 48). However, removal of all divalent cations, by treatment of complete medium with 1mM EDTA, resulted in levels of conjugation little greater than those achieved following treatment with sodium azide (Figure 48).

Figure 46. Adhesion blockade of conjugate formation



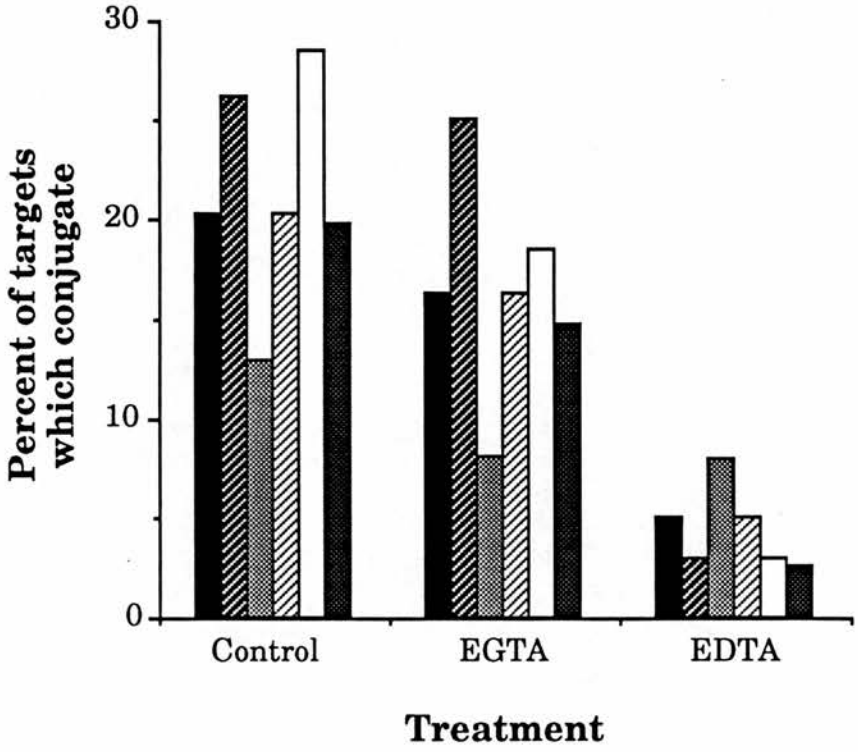
The effect of adhesion blockade on the formation of conjugates between LAK cells and bladder cancer cell lines. Target cells were incubated with saturating concentrations of monoclonal antibodies to ICAM-1 and ICAM-1 and effector cells were incubated with antibodies to LFA-1 for 30 minutes. Control incubations were performed in the presence of antibody diluent. Conjugates were formed by centrifugation and assessed using the flow cytometric methods described. Shown is a representative experiment for each line.

Figure 47. Conjugation is an energy dependent process



The effect of sodium azide (0.05%) on the formation of conjugates between LAK cells and bladder cancer target cells. Bladder cancer cell lines were briefly pre-treated with sodium azide or medium alone prior to the formation of conjugates by centrifugation. The cell lines shown are RT4 (■), RT112 (▨), MGH-U1 (▩), EJ18 (▨), SD (□), UMUC-3 (■), and J82 (▤). A representative experimental value is shown for each cell line.

Figure 48. Divalent cations are important for conjugation



The role of divalent cations was investigated with the aid of calcium chelating agent EGTA (1mM) and divalent cation chelating agent EDTA (1mM). Conjugates were formed in medium containing either EGTA or EDTA , or in complete medium alone (control). Shown are the results for six cell lines; RT4 (■), Rt112 (▨), MGH-U1 (▩), EJ18 (▨), SD (□), and J82 (■).

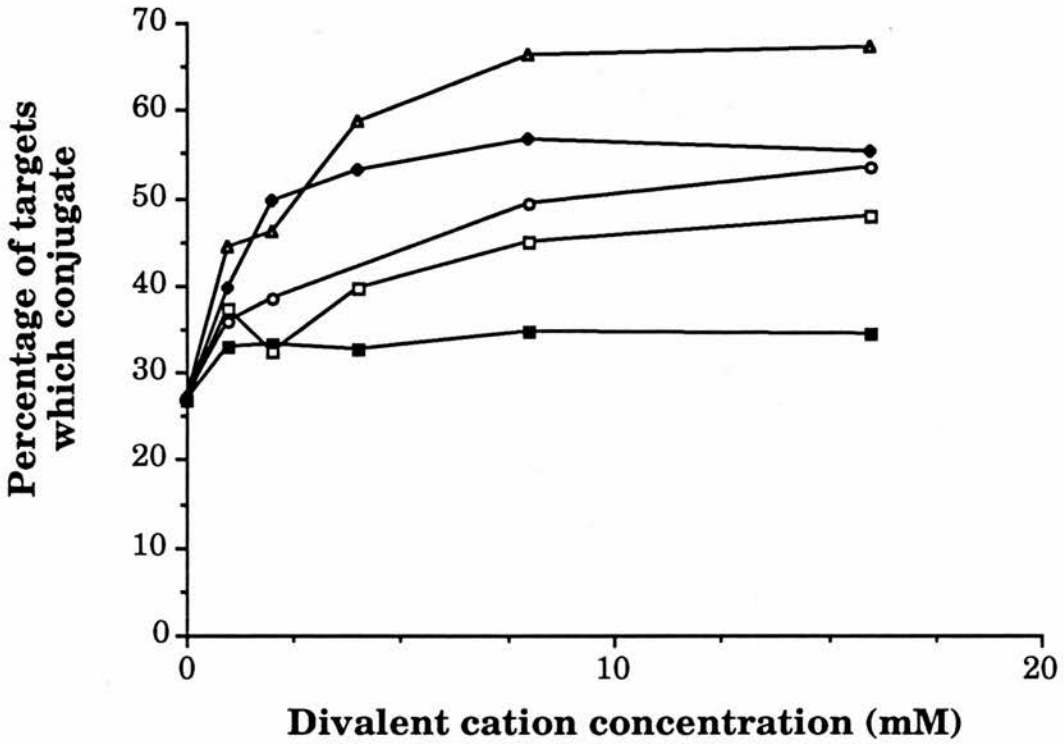
3.3.10 The role of calcium, magnesium and manganese divalent cations in conjugation

The above studies demonstrate the dependence of conjugation upon the presence of divalent cations. Dransfield and colleagues demonstrated that the binding of mAb24 to LFA-1 required the presence of magnesium ions (Dransfield and Hogg 1989) and the work of Figdors group has shown the expression of the L16 epitope is dependent on the presence of calcium cations (Keizer *et al* 1989). In order to further dissect such phenomenon, the effect of increasing the Mg^{2+} or Mn^{2+} concentration in both the presence and absence of Ca^{2+} was investigated.

When the concentration of either calcium, magnesium or manganese in complete medium was increased an increase in the levels of conjugation was observed for the three lines tested (SD, EJ18 and J82, Figures 49, 50 and 51 respectively). The increase in binding was dependent upon the concentration of divalent cation. Optimal binding occurring in the presence of between 4 and 8mM of each of the cations investigated. In the case of SD and EJ18, the presence of manganese cations resulted in an increase in binding grater than in the presence of magnesium or calcium. This was not the case for J82, increased manganese concentration resulted in little change in the percentage of conjugates formed.

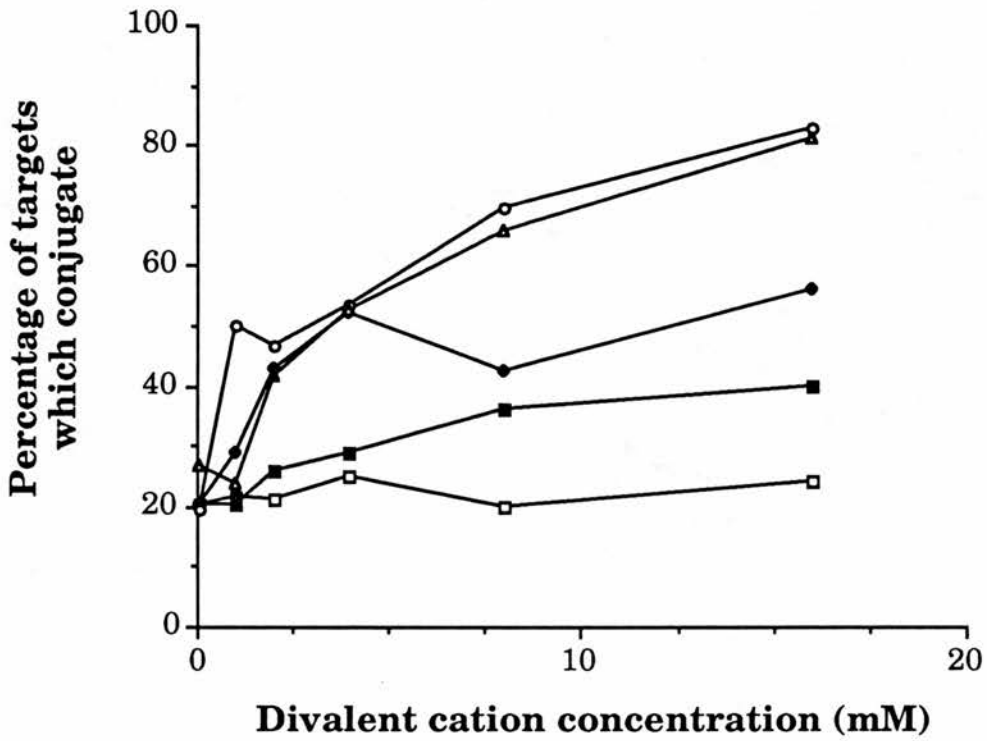
Following removal of calcium ions from complete medium, the addition of magnesium ions had a dramatic effect upon the percentage of targets which formed conjugates with LAK cells (also Figure 49, 50 and 51). As little a 1mM Mg^{2+} resulted in a significant increase in the level of conjugation above control. Maximal conjugation was seen with between 8 and 16 mM Mg^{2+}. The level of conjugation in the presence of magnesium

Figure 49. Conjugation is dependent upon divalent cations



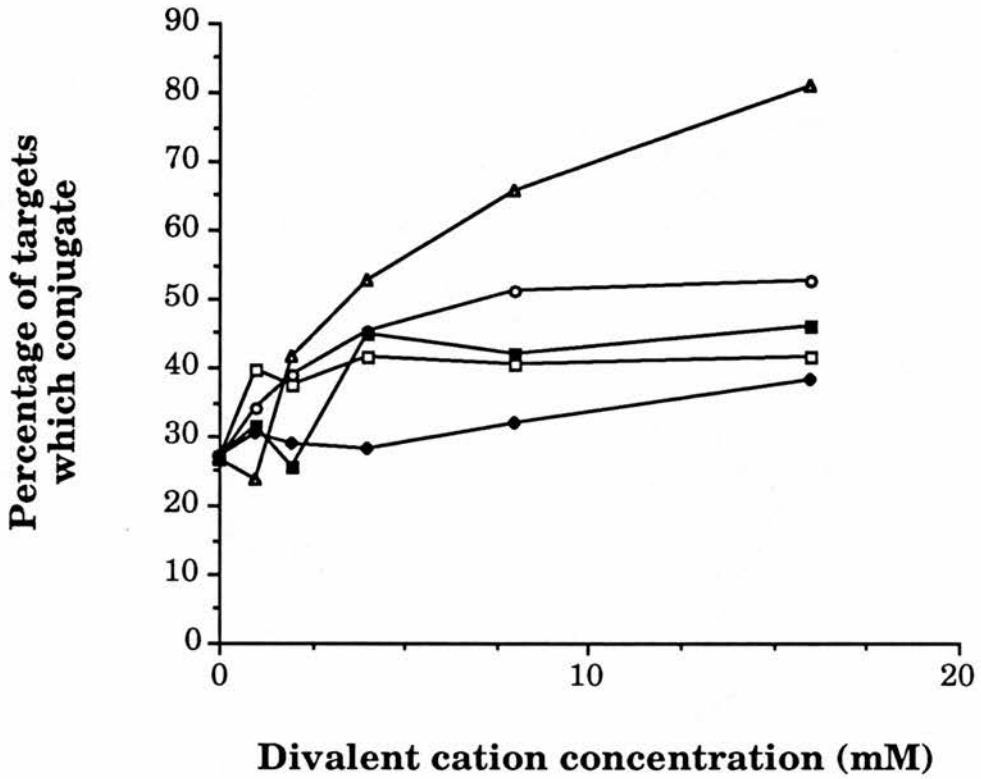
The effect of different divalent cations in the presence and absence of EGTA on the formation of stable conjugates between the cell line SD and LAK cells. Conjugates were formed in RPMI containing the indicated concentrations of either calcium (▫), magnesium (■), manganese (•), or containing EGTA + magnesium (○), or EGTA + manganese (▲). Shown is a representative experiment.

Figure 50. Conjugation is dependent upon divalent cations



The effect of different divalent cations in the presence and absence of EGTA on the formation of stable conjugates between the cell line EJ18 and LAK cells. Conjugates were formed in RPMI containing the indicated concentrations of either calcium (▫), magnesium (■), manganese (●), or containing EGTA + magnesium (○), or EGTA + manganese (▲). Shown is a representative experiment.

Figure 51. Conjugation is dependent upon divalent cations



The effect of different divalent cations in the presence and absence of EGTA on the formation of stable conjugates between the cell line J82 and LAK cells. Conjugates were formed in RPMI containing the indicated concentrations of either calcium (□), magnesium (■), manganese (●), or containing EGTA + magnesium (○), or EGTA + manganese (▲). Shown is a representative experiment.

but in the absence of calcium was far greater than in the presence of calcium, suggesting competition between these two ion species.

The maximal degree of conjugate formation was observed in the presence of manganese following removal of calcium ion using EGTA (1mM). Treatment with between 4 and 16mM manganese increased conjugation by over 100% for all three cell lines investigated.

3.3.11 Calcium ions compete with magnesium ions for binding to LFA-1

In order to investigate the relationship between Mg^{2+} and Ca^{2+} in conjugate formation, a competition study was employed. The addition of 1mM Ca^{2+} to complete medium containing 1mM EGTA and 4mM Mg^{2+} resulted in almost complete negation of the increased binding attributable to Mg^{2+} (Figure 52). Further increases in calcium ion concentration resulted in a progressive decrease in conjugate formation reaching baseline levels with 16mM calcium ions.

3.3.12 Manganese increases conjugate formation by an LFA-1 dependent mechanism

The mechanism by which manganese increases conjugate formation was investigated. As LFA-1 is known to contain several cation binding sites, one possibility was that manganese ions were binding directly to LFA-1 thereby leading to conformational changes. Another possibility was that Mn^{2+} was increasing conjugation by an LFA-1 independent mechanism.

When conjugates were formed in the absence of Ca^{2+} and in the presence of Mn^{2+} elevated levels of conjugation were observed. When cells

were also treated with EGTA and Mn^{2+} and conjugates formed in the presence of sodium azide or anti-CD11a antibodies, a reduced levels of conjugation was observed (Figure 53). This indicated that the increased binding due to manganese was at least partially due to changes in LFA-1.

3.3.13 Calcium does not compete with manganese for binding to LFA-1

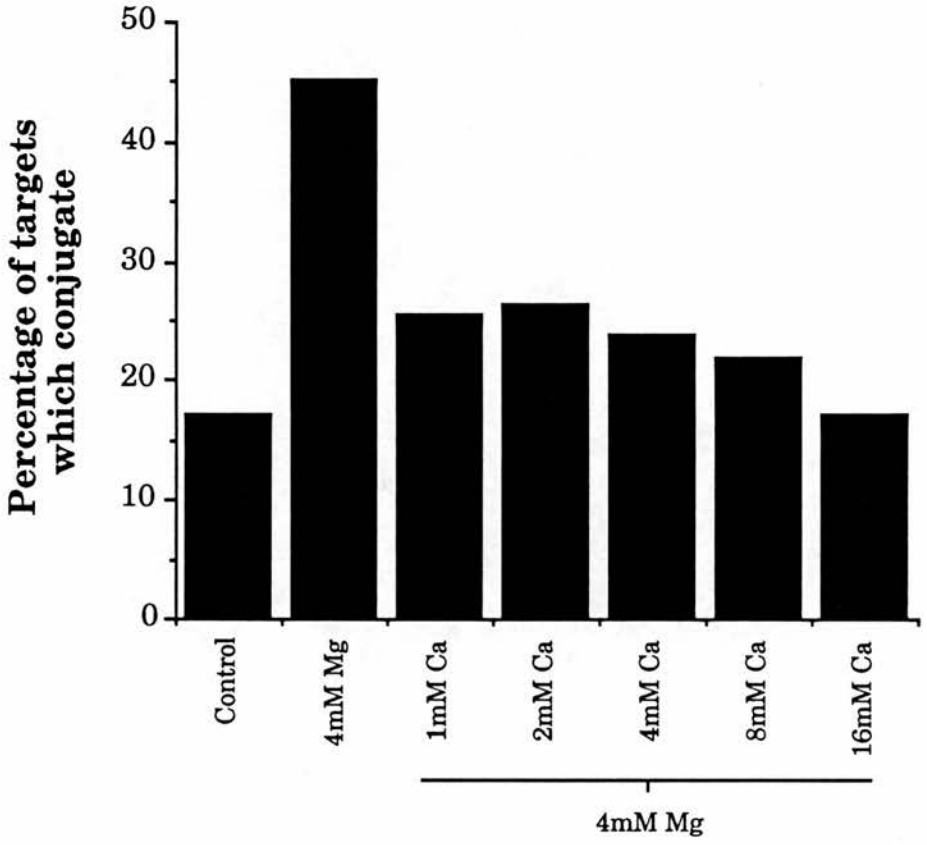
Calcium competes with magnesium ions thereby lowering the binding capacity of cells to the level observed in controls. This did not appear to be the case for manganese as calcium ions were unable to compete for binding (Figure 54). Interestingly this result contrast with that obtained for manganese ions in the absence of EGTA (1mM) (Section *3.3.10*).

3.3.14 Treatment with IFNγ increases the ability to form conjugates

Previously it has been demonstrated that following 24 hours stimulation of target cells with IFNγ their susceptibility to LAK mediated cytolysis was increased. Furthermore, it has been shown that IFNγ induces and augments the expression of the intercellular adhesion molecule ICAM-1 on bladder cancer cells. In order to investigate the mechanism by which IFNγ leads to increased susceptibility to LAK, and to confirm the role of augmented ICAM-1 expression, cells were stimulated with IFNγ for 24 hours prior to assay in the described conjugation assay.

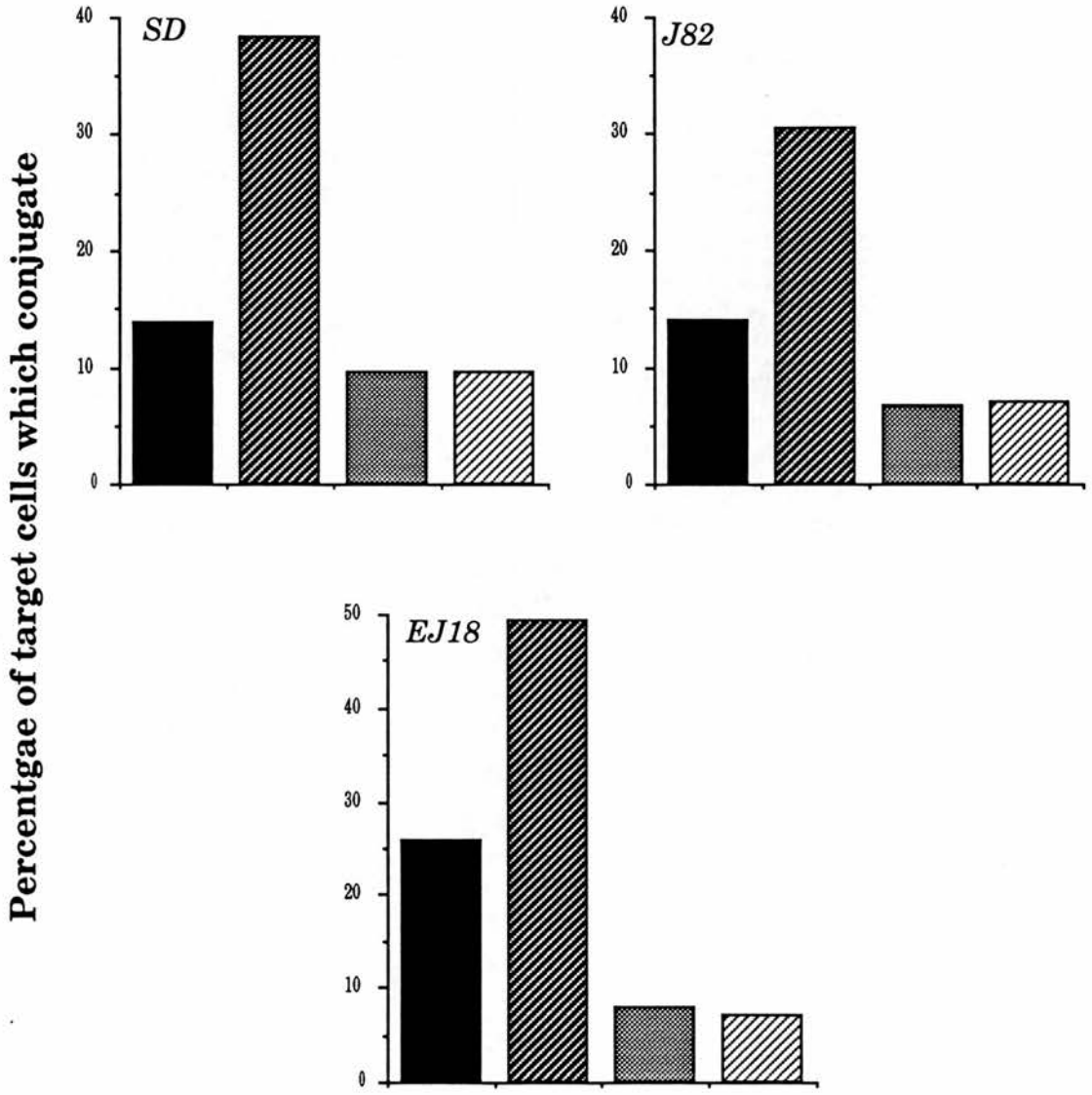
Following stimulation of bladder cancer cell lines with $100Uml^{-1}$ IFNγ for 24 hours an increase in the percentage of targets cells with which effector cells had conjugated was observed (Figure 55).

Figure 52. Calcium competes with magnesium for binding to LFA-1 on LAK cells



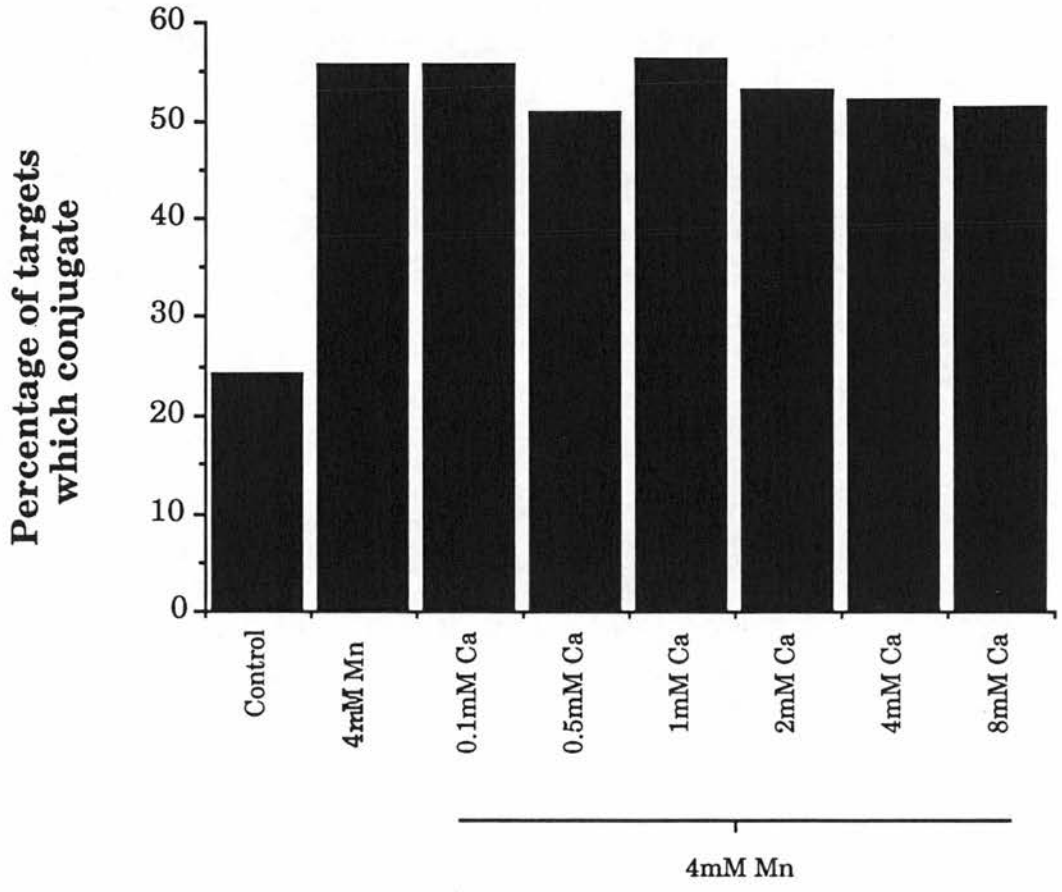
The competition between calcium and magnesium ions for LFA-1 cation binding sites. Cells were washed in medium containing the indicated concentrations of magnesium or calcium ions prior to determination of the percentage of conjugates. All tests except control were performed in the presence of 1mM EGTA.

Figure 53. Manganese increases conjugation by an LFA-1 dependent mechanism



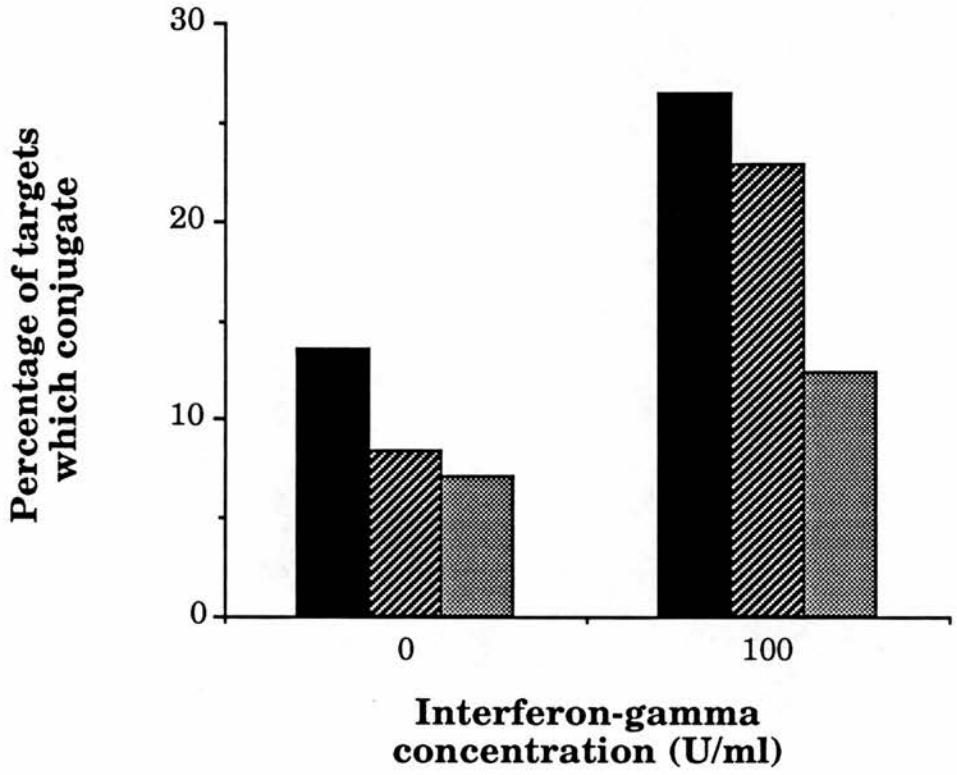
The mechansim by which manganese ions increase conjugation was investigated by treating cells with agents known to inhibit LFA-1 dependent conjugation. Cells were either untreated (■), treated with EGTA and 4mM manganese (▨), treated with EGTA, 4mM manganese and sodium azide (0.05%) (▩), or treated with EGTA, 4mM manganese and antibodies to CD11a (▨). Conjugation was determined after brief centrifugation and resuspension. The data shown are those for three bladder cancer cell lines.

Figure 54. Calcium does not compete with manganese for binding to LFA-1 on LAK cells



The competition between calcium and manganese ions for LFA-1 cation binding sites. Cells were washed in medium containing the indicated concentrations of magnesium or calcium ions prior to determination of the percentage of conjugates. All tests except control were performed in the presence of 1mM EGTA.

Figure 55. Pretreatment with interferon-gamma increases the number of conjugation events



Cells were pretreated for 24 hours with IFNγ and the ability to form conjugates was comared to that of untreated cells. The three cell lines used were RT4 (■), RT112 (▨), and MGH-U1 (▩).

3.4 The expression and function of interferon-gamma receptors

3.4.1 Positive and negative controls for Scatchard analysis

Prior to the more detailed analysis of receptor expression by bladder cancer cell lines a number of control experiments were carried-out in order to determine the specificity of the assay technique. Negative controls, in the form of normal human erythrocytes, and positive controls, in the form of human cells which had previously been assessed for IFNγ receptor expression (Daudi cells) were used. Scatchard analysis was carried out using $1x10^6$ cells per test in a competitive assay system as described in materials and methods.

No specific binding of IFNγ to erythrocytes could be demonstrated and therefore the cells were presumed not to express the receptor in question. Specific binding was demonstrated by the Daudi cell line and subsequent Scatchard analysis of the binding data showed the expression of high affinity receptors for IFNγ. The mean K_d was 0.6 $x10^{-10}$M (Range 0.5-0.8) and the mean number of receptors per cell was 4,200 (range 2,800-6,100). The results are in broad agreement with those of other workers who reported K_d=5 $x10^{-10}$M and B_{max}=3,500-7000 for the receptor on Daudi (Merlin *et al* 1985).

3.4.2 The number of cells used in Scatchard analysis is critical to the evaluation of receptors

Having confirmed the specificity of the assay technique, the optimum concentration of cells for competitive assay was investigated. A fixed amount of radiolabelled ligand (0.3 ng) was incubated with increasing

numbers of cells (0-2x10^6) in triplicate. Following a single wash, the radioactive content of the cellular pellet was determined. The binding curves for 4 bladder cancer cell lines are shown in figure 56. Specific binding increased with the increasing number of cells. Although the maximal number of counts bound was observed with the largest number of cells, this value was not chosen for subsequent experiments. The number of cells which specifically bound approximately half the maximum number of counts was used for further studies. For all four cell lines this value was determined to be 2x10^5 cells. Unlike specific binding, non-specific binding increased in a linear fashion with increasing numbers of cells.

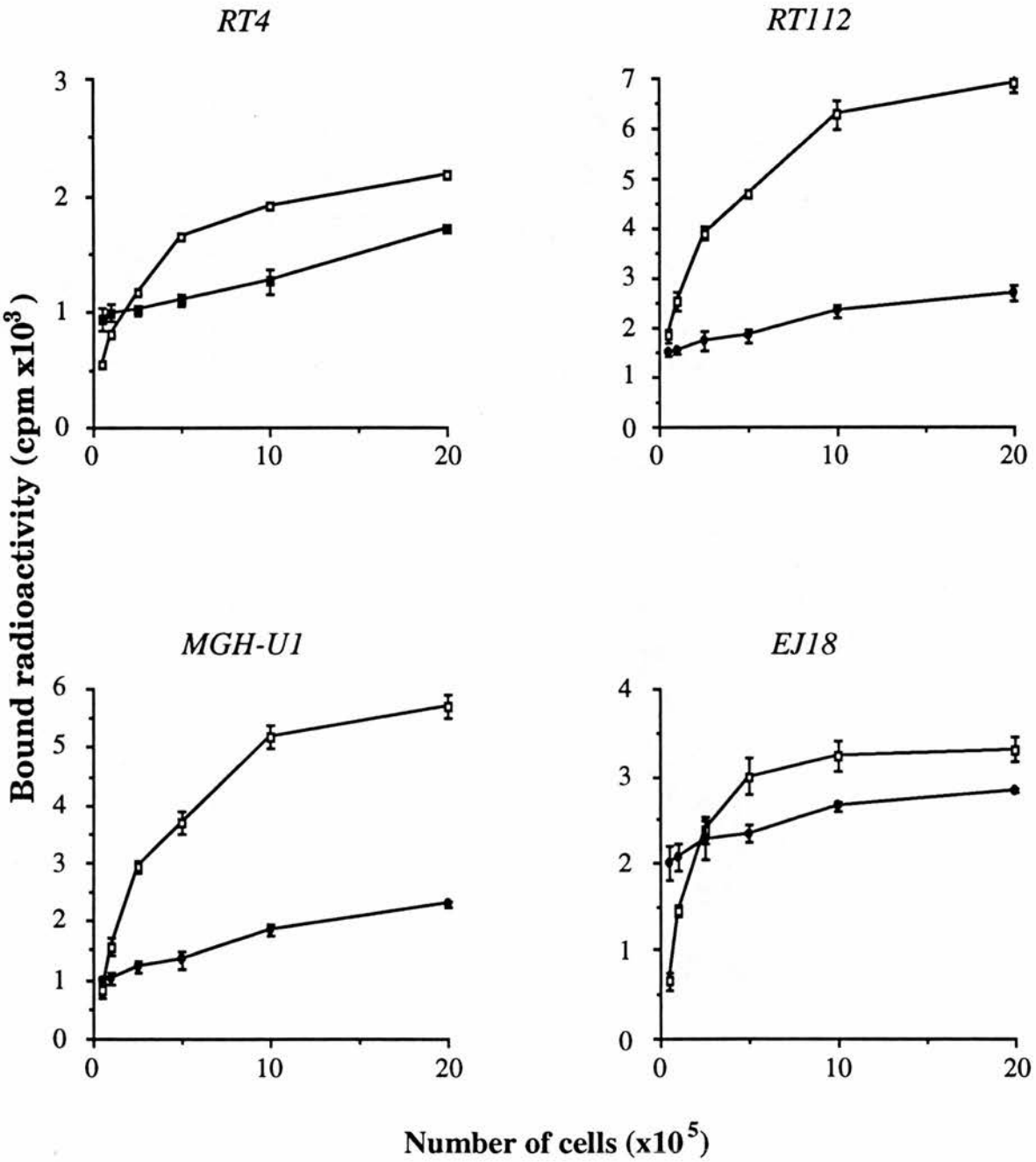
3.4.3 The duration of the assay is an important factor in determining receptor expression

The kinetics of IFNγ binding to the cells was dissected at 4°C. Binding was rapid with over 75% maximal binding occurring within 15 minutes of assay (Figure 57). After 120 minutes no further increase in specific binding was observed. From this data an assay duration of 120 minutes was chosen for subsequent competitive binding studies.

3.4.4 Minimizing non-specific binding in Scatchard analysis

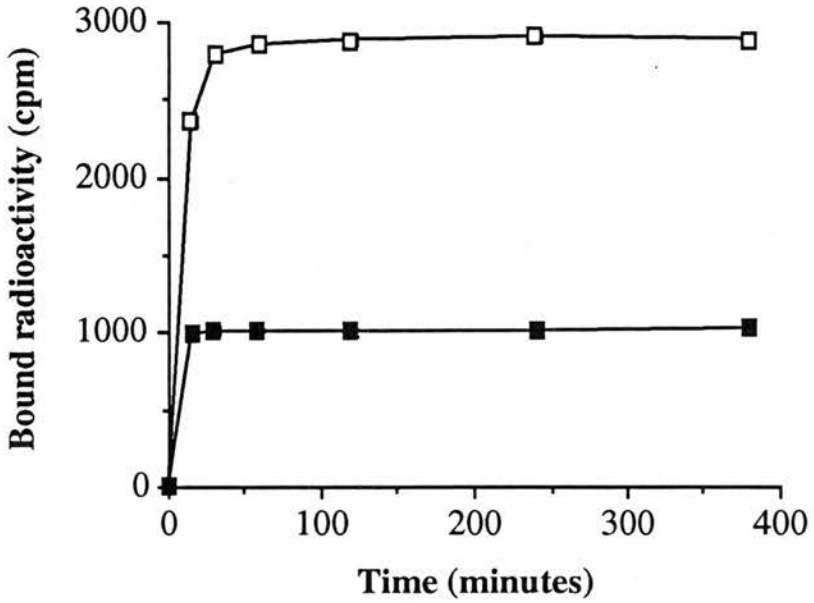
The effect of washing on the NSB following the equilibration of ligand binding was determined. The aim of this experiment was to maximize the difference between the NSB value and the specifically associated interferon-gamma. Figure 58 shows the results obtained for the RT4 cell line. Although a clear difference exists between specifically bound IFNγ and NSB after zero washes, this difference was improved following one

Figure 56. The number of cells is important for analysis



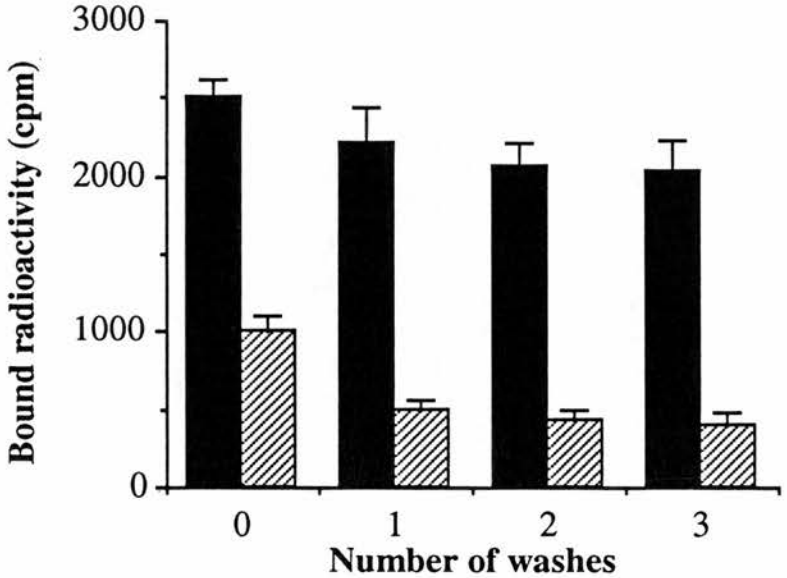
The effect of increasing numbers of cells on the binding of a fixed amount of ligand. Increasing numbers of bladder tumour cells were incubated for 4 hours with 0.3ng of radiolabelled interferon-gamma. Following a single wash the radioactive content of the pellet was counted. Specific binding is represented by the open symbols (□), and non-specific binding by the closed symbols (■). Results are the mean of triplicates and error bars represent one standard deviation.

Figure 57. The kinetics of ligand binding



The kinetics of binding of iodinated interferon-gamma to bladder cancer cells (RT4). Cells were incubated with a fixed amount of radio-ligand for varying lengths of time, following which the cells were centrifuged and the supernatant discarded. The radioactive content of the pellet was assessed and the results presented represent the mean± sd. Specific binding is shown by the open symbols (□), non-specific binding by the closed symbols (■).

Figure 58. The effect of washing on NSB levels



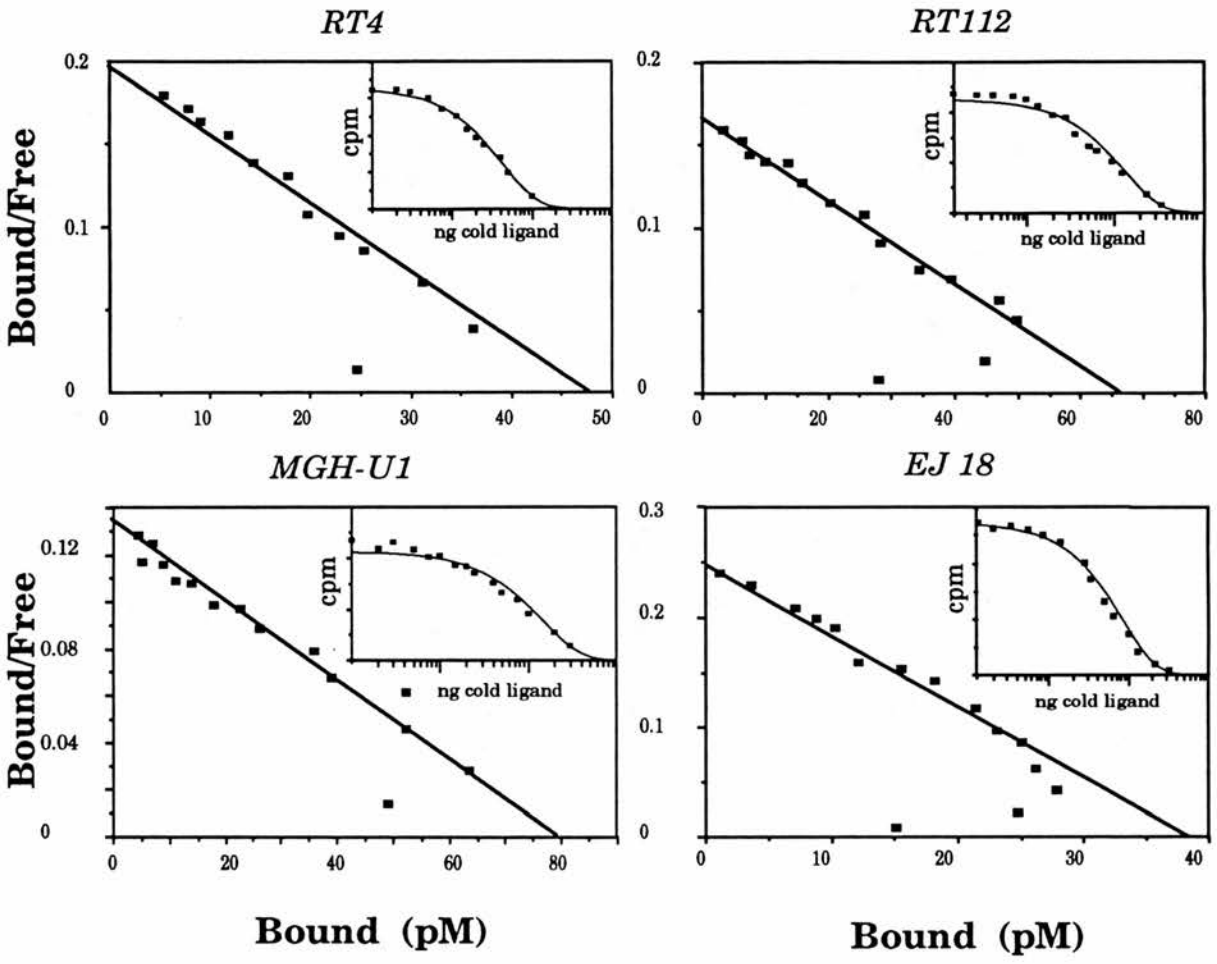
Determination of the optimum number of washes to be given to cells after a competitive binding assay. Cells were incubated for 2 hours with 0.3ng of radiolabelled interferon-gamma. The tubes were centrifuged, supernatant removed, and the radioactive content of the pellet was determined. The pellet was then resuspended in washing buffer and recentrifuged prior to counting. This was repeated up to three times. Solid bars represent specific binding, hatched represent non-specific binding. Results are the mean of triplicates ±1SD.

wash. Further washes, however, failed to result in further improvements in the difference between specifically associated IFNγ and NSB. From this data a single wash was chosen for use in the competitive binding studies.

3.4.5 The Scatchard analysis of interferon-gamma receptors expressed by bladder cancer cells

Using a competitive binding assay the number of IFNγ receptors per cell and their relative affinity for ligand was determined. All four cell lines tested displayed large numbers of high affinity receptors for IFNγ. Representative binding curves and the derived Scatchard plots are shown for the four cell lines studied (Figure 59). From the Scatchard plots a regression line was calculated the correlation coefficients of which resulted in significance values of $p<0.05$ - $p<0.001$. The binding of radiolabelled IFNγ could be displaced by a 100 fold excess of unlabelled ligand. The non-displacable binding ranged between 7 and 27% of the total bound, the upper levels of this being exhibited by the RT4 cell line. The four cell lines had an average K_d of between 1 and 4 x 10^{-10} M and a mean number of receptors per cell of between 19,000 and 37,000 (Table 8). Although marked differences in the mean dissociation constant and number of receptors expressed by some of the cell lines existed, (EJ18<RT4; $p<0.02$), some of the differences failed to reach statistical significance (EJ18<MGH-U1; $p>0.2$, MGH-U1<RT4; $p>0.2$). These differences were not found to correlate with biological response.

Figure 59. The Scatchard analysis of IFNγ receptor



Representative Scatchard plots (main graphs) and the binding curves from which they were derived (inserts) for four bladder cancer cell lines. A linear regression line has been calculated for each Scatchard plot, each point on which represents the mean of a triplicate. Briefly, increasing amounts of unlabelled IFNγ were added to a fixed amount of labelled IFNγ and incubated with $2x10^5$ cells for 120 minutes at 4℃. Each Scatchard plot shows the "tail-back" phenomenon, the likely cause of which is due to the over estimation of NSB. From the gradient of the line the dissociation constant (K_d) is calculated and B_{max} gives the number of receptors per cell.

Table 8. Summary of the results obtained from Scatchard analysis of IFNγ receptor

Cell Line	Number of Receptors per cell	Mean dissociation constant (Kd x10 $^{-10}$ M)
RT4	36,859 ± 20,000	3.6 ± 1.3
RT112	26941 ± 9000	3.3 ± 1.0
MGH-U1	40,812 ± 22,000	4.1 ± 2.0
EJ18	19,827 ± 3,800	1.1 ± 0.6

The table details the results of Scatchard analysis for the interferon-gamma receptor as expressed by four bladder cancer cell lines. The number of receptors per cell was calculated from B_{max} and it was assumed that the moiety of interferon-gamma which bound the receptor was a dimer. The dissociation constant was calculated from the gradient of the linear regression from the Scatchard plot. All data represent the mean of at least six independent determinations, each in triplicate. Results are presented as mean ± sd.

3.4.6 The kinetics of ligand internalization and degradation following receptor binding

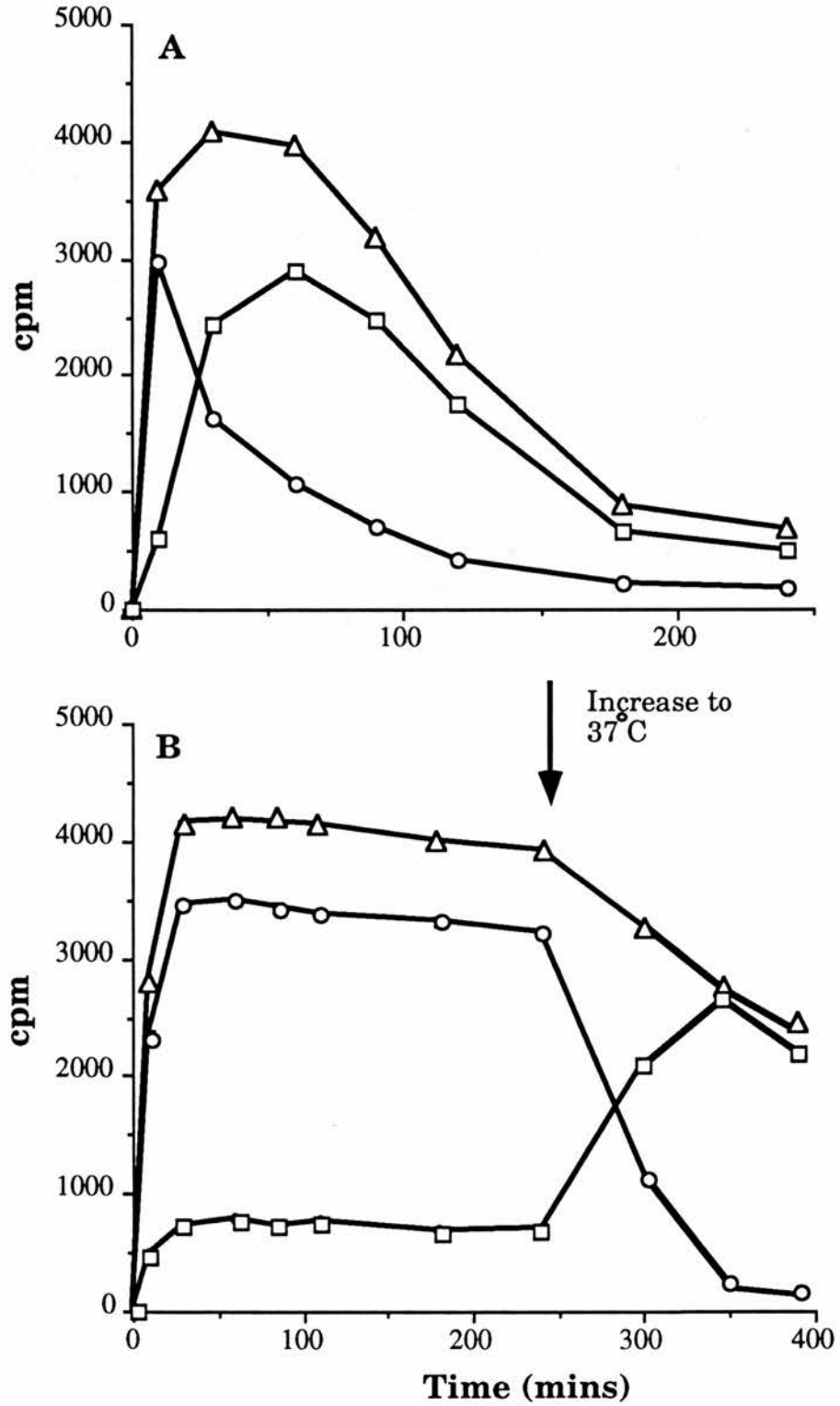
As the differences in biological response of the four cell lines to IFNγ could not obviously be accounted for simply in terms of their receptor status, the rate of ligand binding and internalisation by the cells at physiological temperature (37°C) was investigated. The cell lines were analysed in suspension (as in the Scatchard studies) and also as adherent monolayers.

The kinetic studies on TCC cells in suspension at 37°C revealed extremely rapid surface binding which was maximal after 10 minutes after which, the amount of surface bound ligand declined rapidly (Figure 60). Following receptor binding, an increase in the concentration of internalized ligand was observed which reached maximum levels after 60 minutes. After this, a steady decline in internalized ligand was observed. The total of cell associated ligand was greatest after 30 minutes and declined thereafter. After 240 minutes total cell associated ligand was less than 20% maximal levels. Figure 58 shows representative kinetics for MGH-U1 in suspension at 37°C, the other three cell lines followed similar patterns. At 4°C surface binding was again a rapid event but was not followed by a marked degree of ligand internalisation. Only when the temperature was increased to 37°C was the ligand internalised (Figure 61).

The rate of binding and internalization by adherent cells was different to that of cells in suspension. The kinetics of surface binding were less rapid with half maximal binding occurring after 10 minutes (Figure 62). This proceeded to a maximum after 60 minutes with all lines except RT4 (maximum 100 minutes), following which a gradual decrease in the

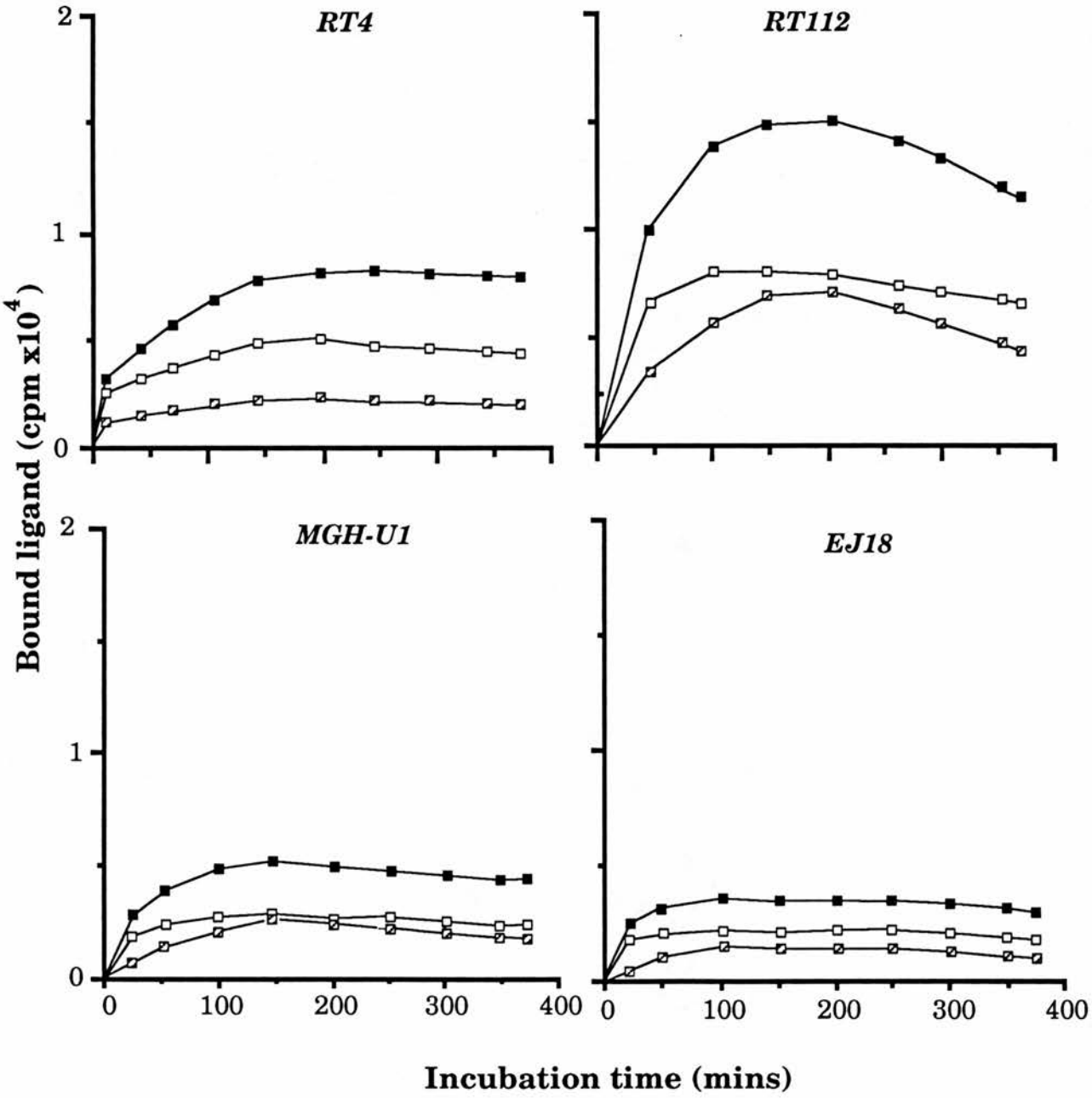
amount of surface bound ligand was observed. Surface binding was followed by a steady increase in the levels of internalized ligand. Levels of internalized ligand continued to increase until 180 minutes in all lines except RT4, where the increase continued until 300 minutes. Significant differences were apparent between the maximum amount of ligand bound by the cell lines; MGH-U1 5,124±497cpm, EJ18 3,875±1022cpm, RT4 7,989±648cpm, RTR112 15,661±1720cpm (MGH-U1<RT4<RT112, $p<0.02$). MGH-U1 and EJ18 (G3) both bound the least ligand. The two lower grade tumour lines maximally bound higher levels of ligand, with RT112 (G2) binding the highest.

Figures 60 & 61. The binding of IFNγ to MGH-U1 cells



The kinetics of IFNγ binding and internalization by MGH-U1 at two different temperatures (A-37, B-4). Cells were incubated with IFNγ for the indicated times and surface bound ligand was removed using acidic buffer. After centrifugation pellet and supernatant associated radioactivity were determined separately. In figure B the temperature was increased to 37°C after 240 minutes. Shown is the amount of surface bound ligand (o), internalized lignad (□), and the total cell associated ligand (Δ).

Figure 62. The rate of binding of IFNγ to four TCC cell lines



The kinetics of cell surface binding, internalization and degradation of interferon-gamma by four bladder cancer cell lines. Cells in suspension were incubated with 0.3ng of radiolabelled IFNγ for the indicated periods. Cell surface ligand was removed by acid stripping techniques and separated from the pellet by centrifugation. The cell associated and supernatant associated radioactive content were determined separately, the addition of both giving the total cell associated ligand. The experiments were carried out in triplicate and standard deviations were within 5%. The total cell associated ligand (■), the receptor bound ligand (□), and the internalized ligand (▨) are depicted.

3.5 The signal transduction of the interferon-gamma receptor

3.5.1 Induction of ICAM-1 and HLA class II molecule expression.

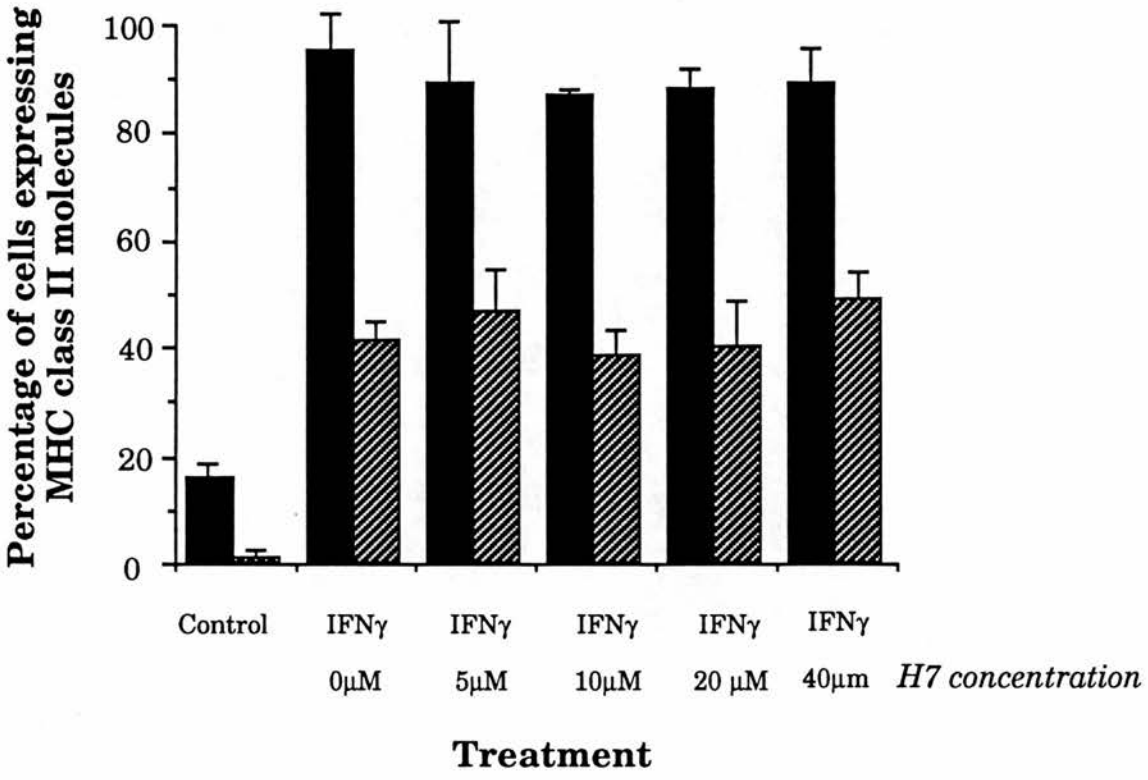
The constitutive expression of these molecules varies between cell lines. Following incubation with IFNγ further expression of both class II and ICAM-1 molecules can be induced. However, cell lines which respond well in terms of class II induction do not necessarily respond well with ICAM-1 induction, or *vice versa*. The mechanisms of signal transduction from the IFNγ receptor are not clearly defined, major differences being reported by different groups. The mechanisms of signal transduction were investigated in terms of HLA class II and ICAM-1 expression with the aim of firstly determining any differences between cell lines, and secondly any differences between class II and ICAM-1 expression induced by IFNγ.

3.5.2 The role of PKC in IFNγ induced HLA class II expression.

Protein kinase C is a common intracellular messenger, the role of which was investigated using specific inhibitors and activators of its function. The IFNγ induced class II molecule expression on RT4 and RT112 was not inhibited by a PKC inhibitor H-7 (1-40μM) (Figure 63).

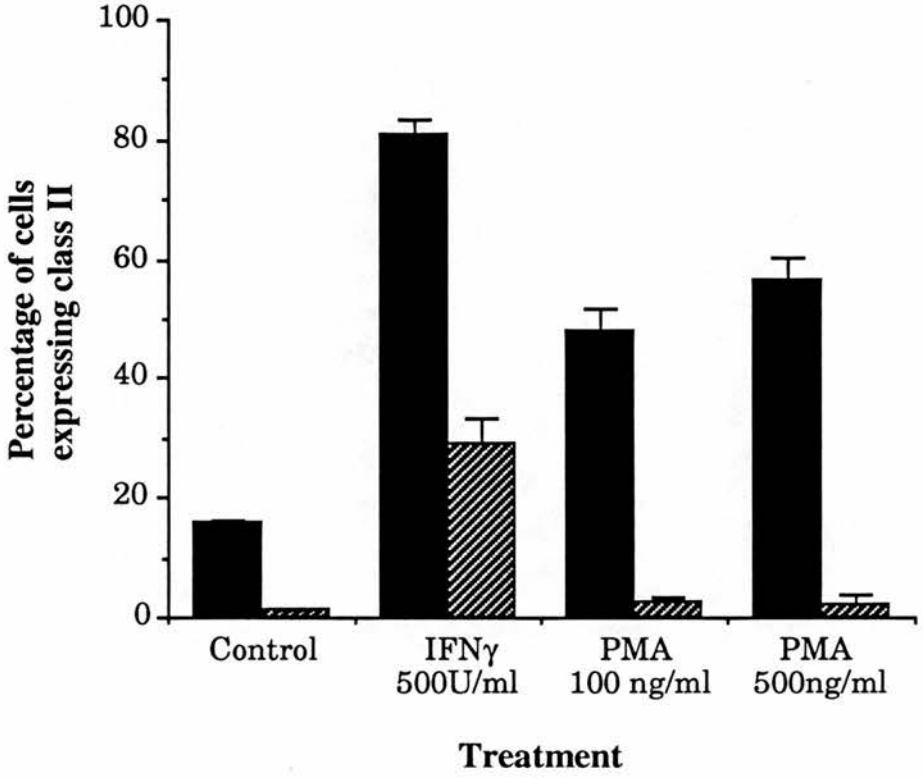
The second step in the investigations concerning PKC involved activation via PMA. When RT4 cells were treated with PMA at either 100 or 500ngml^{-1}, increased expression of HLA class II was observed, however, no similar induction was observed with RT112 (Figure 64). The increased class II expression by RT4 resulting from treatment with PMA was less than that achieved with IFNγ, suggesting the involvement of other factors in optimal signal transduction.

Figure 63. An inhibitor of PKC fails to block IFNγ induced class-II expression



The effect of protein kinase C inhibitor H7 on the expression of MHC class II molecules induced by interferon-gamma. Cells were stimulated for 48 hours with 500U/ml IFNγ and increasing concentration of H7. Control values are those obtained in the absence of IFNγ. MHC class II expression was determined by flow cytometry. The two bladder cancer cell lines shown are RT4 (■), and RT112 (▨). Error bars represent 1 standard deviation.

Figure 64. Treatment with PMA can induce class II expression



The effect of activation of PKC via treatment of cells with PMA. Cells were incubated with either medium alone (control), interferon-gamma, or PMA at the concentrations indicated for 48 hours. The expression of HLA class II molecules was determined by flow cytometry. The cell lines used were RT4 (■), and RT112 (▨). Error bars show 1 standard deviation.

3.5.3 The effect of calcium ionophore on HLA class II expression

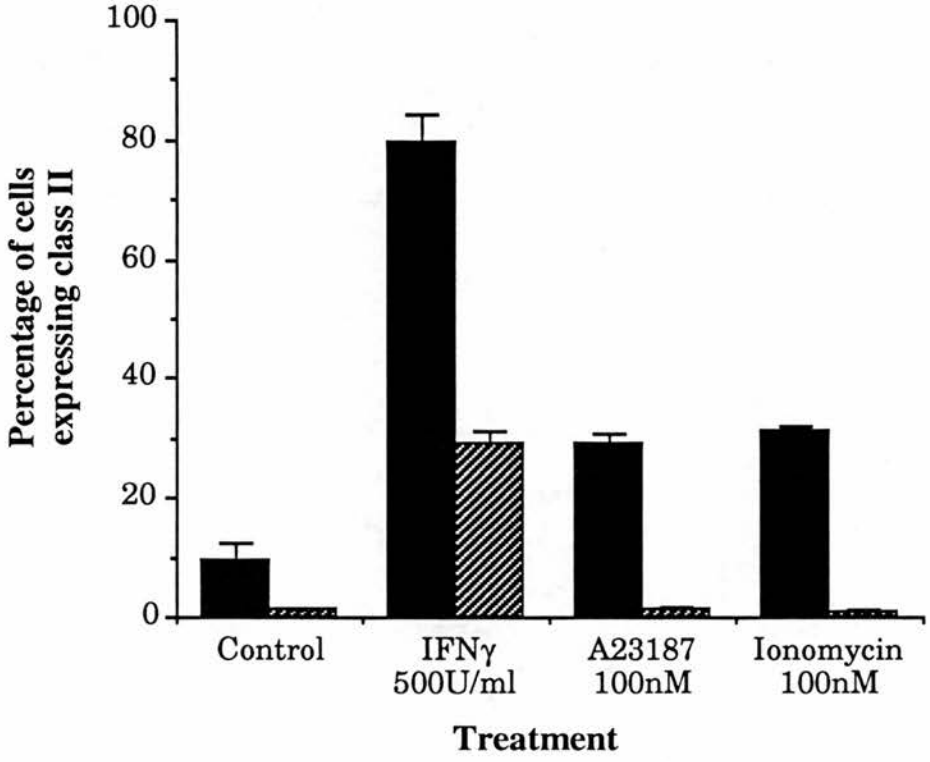
A further important second messenger pathway involves the activation of calcium/calmodulin dependent protein kinases. When RT112 cells were treated for 48 hours with the calcium ionophores A23187 and ionomycin, no change in class II expression was observed. However, the expression of class II antigens on RT4 was induced following stimulation with calcium ionophore (Figure 65).

Several workers have reported the "cross-talk" phenomenon involvement in the signal transduction of various cytokine receptors. This involves two (or more) signalling pathways acting together to generate a response. Simultaneous activation of protein kinase C and an increase in intracellular calcium by PMA and ionomycin or A23187 failed to result in increased expression of HLA-DR by either RT4 or RT112 (data not shown).

3.5.4 Effect of inhibition of calmodulin-dependent activity on IFNγ-induced Ag expression.

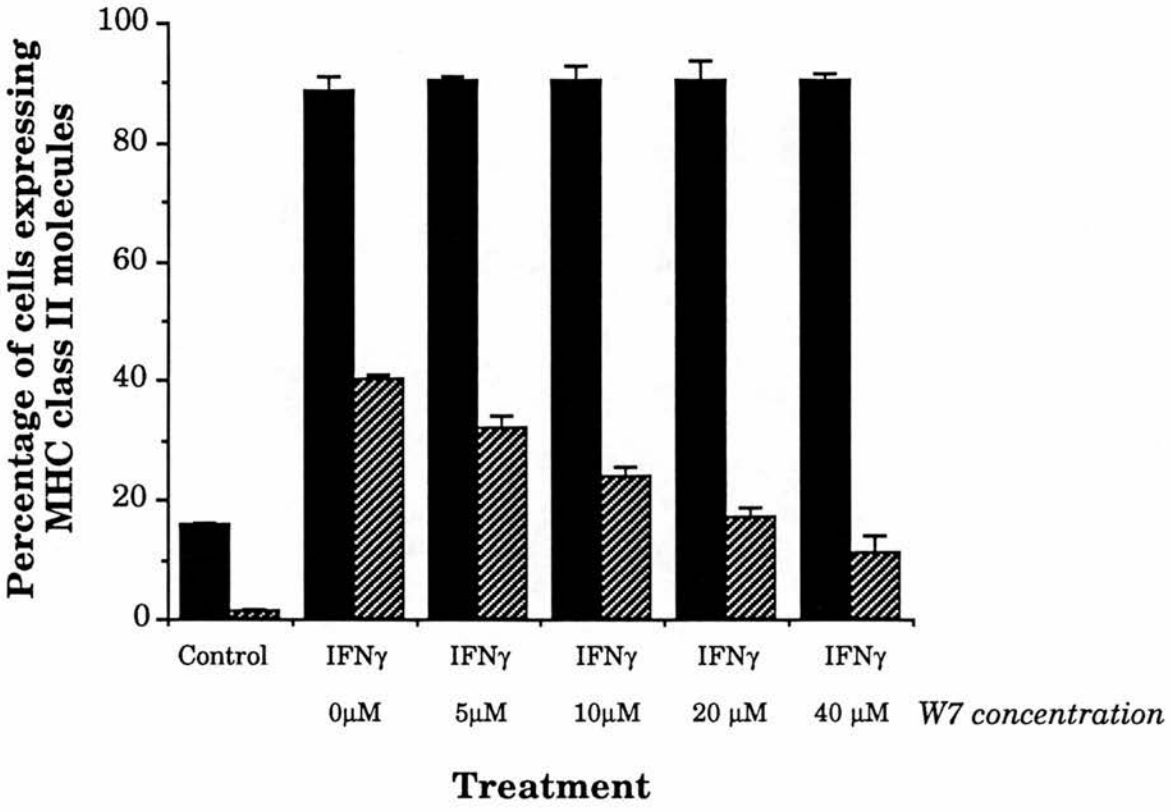
Because calmodulin can be activated by an increase in intracellular calcium ($[Ca^{2+}]_i$), we determined if inhibition of calmodulin dependent processes prevented the induction of surface antigens by IFNγ. When RT112 cells were treated with W-7 (an inhibitor of calmodulin dependent processes) the induction of class II by IFNγ was inhibited in a dose dependent manner (1-40μM) (Figure 66). This was not the case for RT4 cells in which no inhibition of IFNγ induced DR expression was observed.

Figure 65. Calcium ionophore induces HLA class II expression



The effect of calcium ionophores A23187 and ionomycin on the expression of HLA class II molecules by two bladder cancer cell lines RT4 (■) and RT112 (▨). Cells were stimulated for 48 hours with the indicated agents and class II expression determined by flow cytometry. Error bars indicate 1 standard deviation.

Figure 66. The calmodulin inhibitor W7 prevents IFNγ induced HLA class II expression



The effect of calmodulin inhibitor W7 on the expression of MHC class II molecules induced by interferon-gamma. Cells were stimulated for 48 hours with 500U/ml IFNγ and increasing concentration of W7. Control values are those obtained in the absence of IFNγ. MHC class II expression was determined by flow cytometry. The two bladder cancer cell lines shown are RT4 (■), and RT112 (▨). Error bars represent 1 standard deviation.

3.5.5 The role of extracellular calcium in IFNγ induced class II expressions

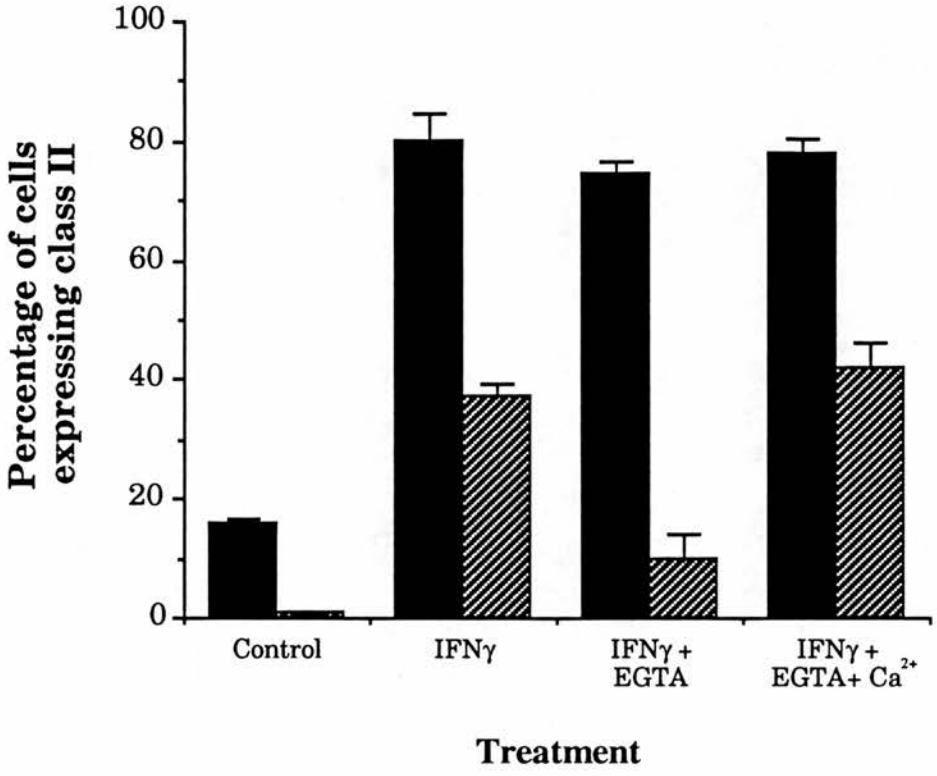
In order to investigate the role of extracellular calcium in IFNγ induced DR expression, extracellular calcium was chelated using EGTA (1 mM). Depletion of extracellular calcium in this manner appeared to inhibit the IFNγ induced MHC class II Ag expression by RT112, suggesting the involvement of calcium influx in its induction, but did not inhibit DR expression by RT4 (Figure 67). The inhibition of expression was not due to the toxic effects of EGTA as replenishment of the calcium concentration to original levels reversed the reduction in class II expression.

3.5.6 Effect of inhibition of calcium transients on the induction of class II expression by IFNγ

To study the functional significance of calcium influx through ion channels, cells were pretreated with diltiazem (1μM) for 30 min and incubated in the presence of diltiazem with IFNγ (500Uml^{-1}) for 48 hr. Diltiazem failed to inhibit the induction of surface class II expression by RT4 and RT112 cells (Figure 68).

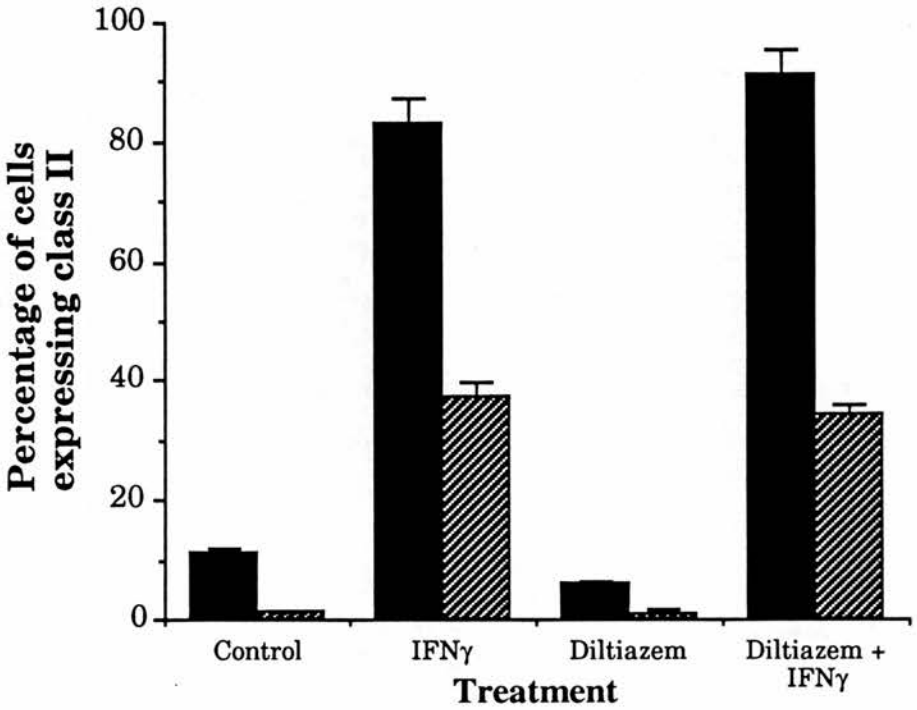
The effect of inhibition of an increase in $[Ca^{2+}]_i$ mobilisation by TMB-8 on IFNγ induced class II expression was determined by pretreating RT4 and RT112 cells with TMB-8 (75 μM) followed by incubation in the presence of TMB-8 with IFNγ (500Uml^{-1}) for 48 hr. TMB-8 partially inhibited the IFNγ induced HLA-DR Ag expression by RT112 cells but not by RT4 (Figure 69). A mean reduction of 53±10% of the level obtained with IFNγ was observed on the RT112 cell line following treatment with TMB-8.

Figure 67. Depletion of extracellular calcium ions prevents IFNγ induced class II expression by RT112



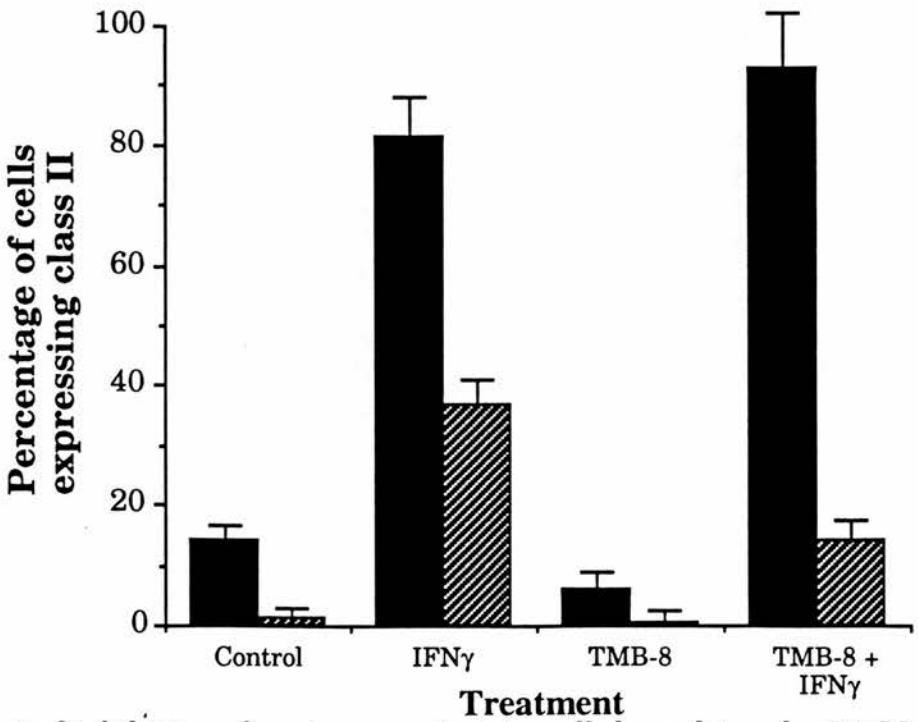
The role of extracellular calcium in the signal transduction of the IFNγ receptor. Extracellular calcium was chelated with 1mM EGTA and the cells were stimulated with 500U/ml of IFNγ for 48 hours prior to the determination of class II expression by flow cytometry. The two cell lines shown are RT4 (■), and RT112 (▨). Error bars show 1 standard deviation.

Figure 68. Calcium channel blockade does not inhibit IFNγ induced HLA class II expression



The effect of calcium channel blockade on IFNγ induced class II expression by RT4 (■), and RT112 (▨). Cells were pre-treated with 1μM diltiazem for 30 minutes prior to stimulation with 500U/ml IFNγ for 48 hours. Antigen expression was determined via flow cytometry.

Figure 69. TMB-8 inhibits IFNγ induced class II expression in RT4



The effect of inhibition of an increase in intracellular calcium by 75μM TMB-8 on IFNγ induced class II expression by RT4 (■), and RT112 (▨). Cells were pretreated with TMB-8 and then stimulated with IFNγ for 48 hours. MHC class II expression was determined by flow cytometry. Error bars represent 1 standard deviation.

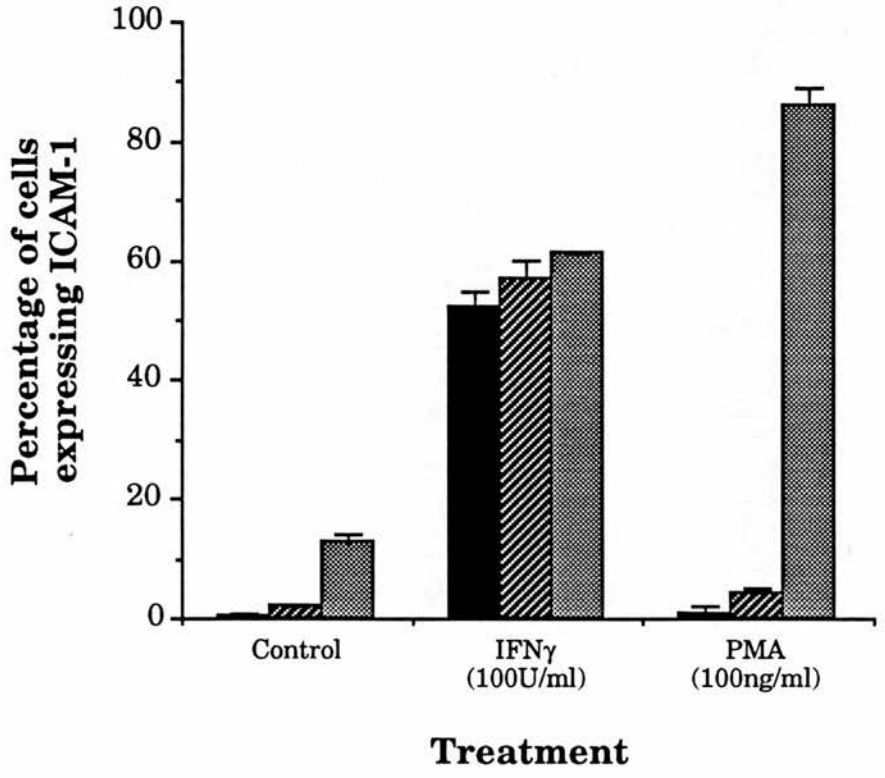
3.5.7 IFNγ fails to induce calcium mobilisation in bladder cancer cells

As pharmacological manipulation of calcium mobilisation appeared to alter the ability of IFNγ to induce class II expression by RT112, we loaded the cells with Fura-2AM, a calcium indicating dye, and subsequently monitored the cytosolic calcium concentration in the absence and presence of IFNγ. The basal levels of calcium concentration for RT4 and RT112 were approximately 100nM. When MGH-U1, RT4 and RT112 cells were stimulated with IFNγ no increase in intracellular calcium concentration was observed. As confirmation of the correct functioning of the system, cells were stimulated with the calcium ionophore ionomycin. Immediately following stimulation with ionomycin, an increase in intracellular calcium was observed (data not shown). The subsequent treatment of the cells with digitonin caused the level of intracellular calcium to fall to baseline (data also not shown).

3.5.8 The role of PKC in IFNγinduced ICAM-1 expression

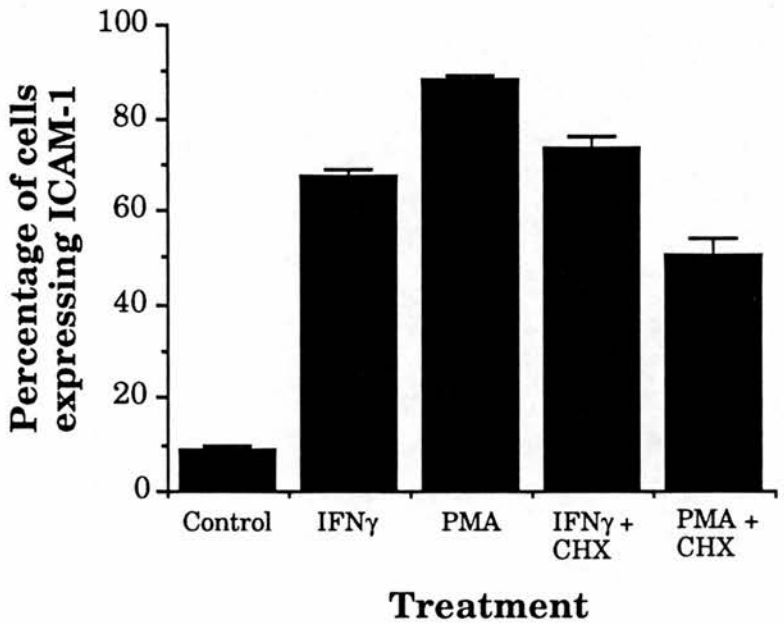
Following 24 hours stimulation with 100ngml^{-1} PMA, the MGH-U1 cell line was observed to express high levels of ICAM-1, however, RT4 and RT112 did not behave in a similar manner (Figure 70). In fact, the levels of ICAM-1 induced by PMA treatment of MGH-U1 were significantly greater than those achieved with the optimal concentration of IFNγ alone ($p<0.02$). Unlike the IFNγ induced antigen expression the PMA inducible ICAM-1 expression was partially inhibited by pretreatment with CHX (1μgml^{-1} for 30 minutes) as shown in Figure 71.

Figure 70. PMA stimulates ICAM-1 expression in MGH-U1 cells



The effect of activation of PKC via treatment of cells with PMA. Cells were incubated with either medium alone (control), interferon-gamma, or PMA at the concentrations indicated for 24 hours. The expression of ICAM-1 molecules was determined by flow cytometry. The cell lines used were RT4 (■), RT112 (▨), and MGH-U1 (▩). Error bars show 1 standard deviation.

Figure 71. The contribution of protein synthesis in PMA induced ICAM-1 expression



The contribution of protein synthesis in PMA induced ICAM-1 expression was assessed using CHX. MGH-U1 were stimulated with either IFNγ (100U/ml) or PMA (100ng/ml) in the absence or presence of CHX (1μg/ml) for 24 hours. Following incubation the expression of ICAM-1 was studied using flow cytometry with monoclonal antibody RR1/1. Bars show the mean of triplicate determinations, error bars indicating 1 standard deviation

3.5.9 Protein kinase C inhibitors H7 and staurosporine fail to inhibit IFNγ induced ICAM-1 expression

Treatment of the MGH-U1 cells with PMA resulted in augmented expression of cell surface ICAM-1 within 24 hours. As PMA is a known activator of PKC, the role of this kinase was investigated with the inhibitor H7 and the specific PKC inhibitor staurosporine.

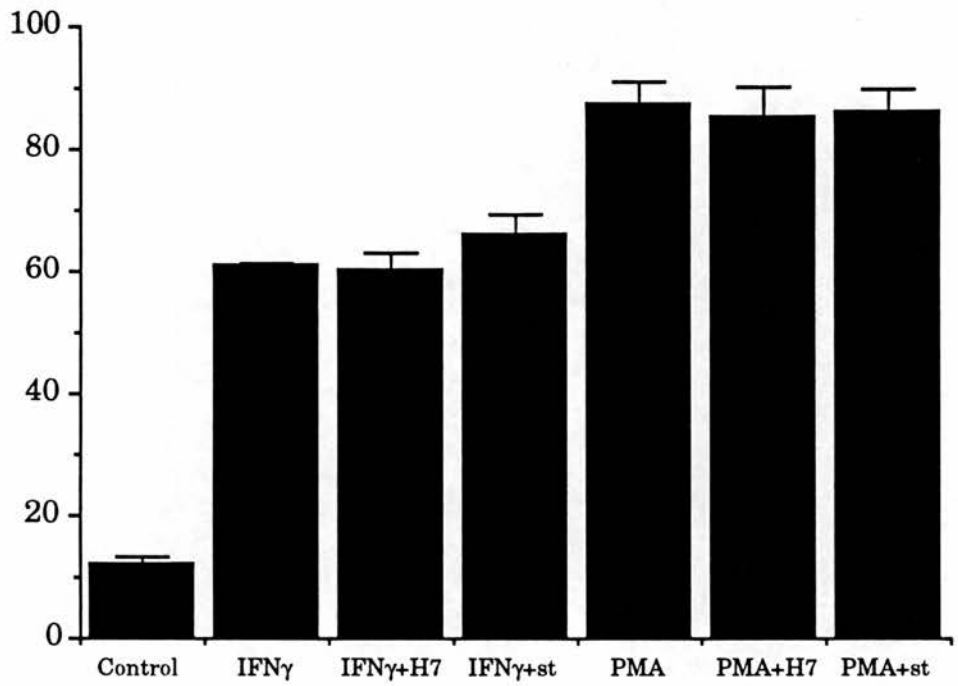
The pharmacological inhibition of PKC did not result in a decreased response to IFNγ (Figure 72). Furthermore, in the case of MGH-U1, when ICAM-1 expression was induced by PMA no inhibition was seen in the presence of staurosporine or H7. This indicated that PKC was not involved in the signal transduction from the IFNγ receptor, and that PMA acts by another mechanism to increase the expression of ICAM-1.

3.5.10 The role of calcium ions in IFNγ induced ICAM-1 expression

The role of divalent calcium ions in IFNγ induced ICAM-1 expression was investigated using calcium ionophores (A23187), calcium chelating agents (EGTA) and drugs known to influence to mobilization of intracellular calcium (TMB-8) or calcium channel function (diltiazem). When the three bladder tumour cell lines were treated with either A23187 or ionomycin for 24 hours no induction or augmentation of ICAM-1 expression was observed (Figure 73).

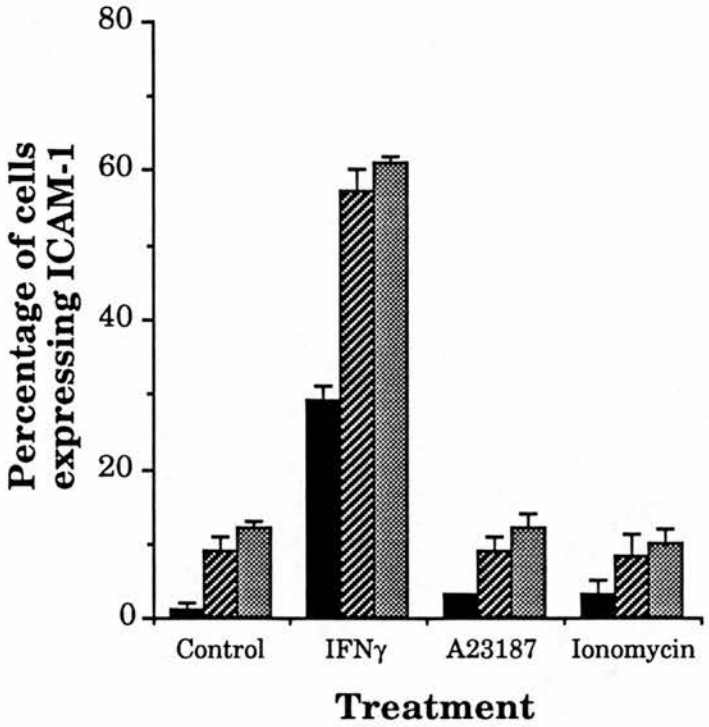
When RT4 and MGH-U1 cells were pretreated with EGTA in order to chelate extracellular calcium prior to stimulation with IFNγ, an increase in ICAM-1 expression was noted (Figure 74), however no change was observed with RT112. Neither blockade of calcium channels (diltiazem) nor inhibition of calcium mobilization (TMB-8) in RT4 caused any change

Figure 72. Inhibition of PKC does not prevent IFNγ or PMA from inducing ICAM-1 expression on MGH-U1



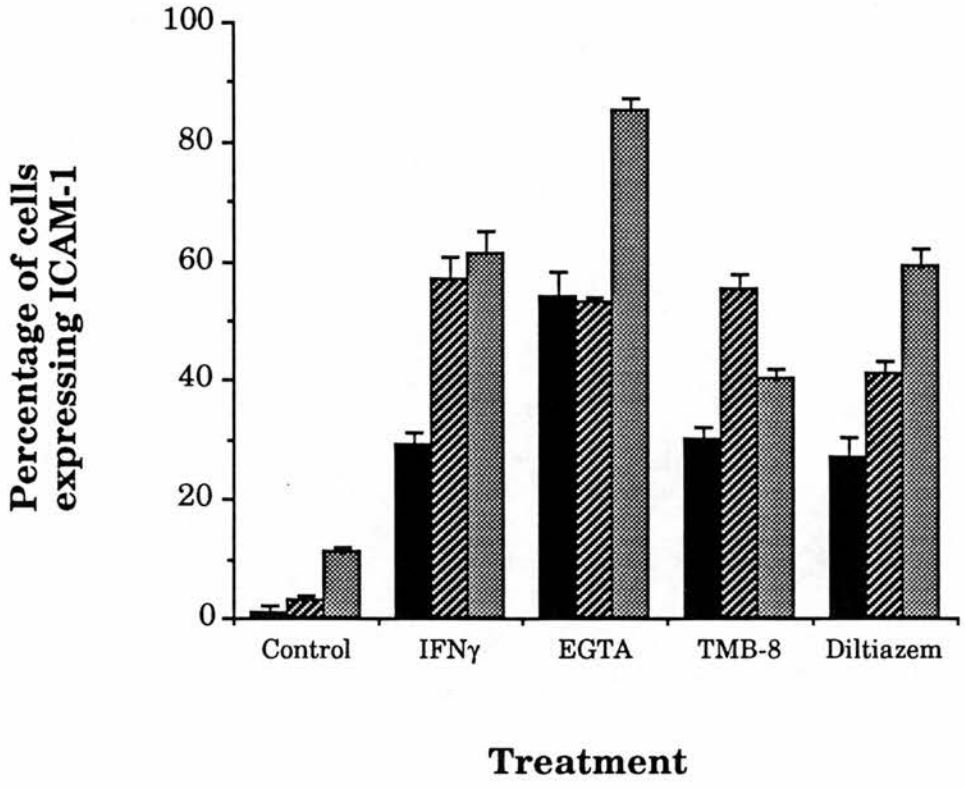
The contribution of PKC to IFNγ and PMA induced ICAM-1 expression was assessed using PKC inhibitors H7 (40μM) or staurosporine (st, 50nM). Cells were first pretreated with these agents prior to stimulation with IFNγ for 24 hours. ICAM-1 expression was determined using flow cytometry with the monoclonal antibody RR1/1. Bar represent the mean of triplicat values, whilst error bars indicate the level of 1 standard deviation.

Figure 73. Calcium ionophores do not induce ICAM-1 expression on bladder cancer cell lines



Cells were treated with either IFNγ (100U/ml) or calcium ionophore (100nM) for 24 hours. The three cells lines used were RT4 (■), RT112 (▨) and MGH-U1 (▩). ICAM-1 expression was determined using flow cytometry with the monoclonal antibody RR1/1. Bars represent the mean of triplicate values, whilst error bars indicate the level of 1 standard deviation.

Figure 74. The role of calcium ions in IFNγ induced ICAM-1 expression on bladder cancer cell lines



Cells were treated with either IFNγ alone (100U/ml) or first pre-treated with EGTA (1mM), TMB-8 (75μM) and Diltiazem (2μM) for 24 hours. ICAM-1 expression was determined using flow cytometry with the monoclonal antibody RR1/1. The three cell lines used were RT4 (■), RT112 (▨) and MGH-U1 (▩). Bars represent the mean of triplicate values, whilst error bars indicate the level of 1 standard deviation.

in ICAM-1 expression (Figure 74). However, channel blockade in the case of MGH-U1 and inhibition of calcium mobilization in the case of RT112 was observed to decrease the response of these cells to IFNγ (Figure 74). Only partial blockade of response was achieved.

3.5.11 The effect of inhibition of calmodulin dependent protein kinase activity on IFNγinduced ICAM-1 expression

The expression MHC class II molecules as induced by IFNγ on RT112 cells was inhibited by W7, an inhibitor of calmodulin dependent protein kinase activity. However, ICAM-1 induction on the same cell line was not inhibited by pretreatment with W7 (10-50μM). Similarly, IFNγ induced ICAM-1 expression on RT4 and MGH-U1 cells was not inhibited by W7 at any concentration (data not shown).

3.6 The relationship of in vitro studies to the situation in vivo

The studies presented so far have examined a variety of parameters associated with the immunotherapy of bladder cancer, the role of various cytokines and the function of adhesion molecules in an artificial killing system. *In vitro* the killing of tumour cells by LAK cells would appear to involve the LFA-1/ICAM interaction as a primary recognition signal between effector and target cell. Tumour cells which express higher levels of the ligand for effector cells would appear to be more susceptible to conjugation and lysis by these cells. Cytokines which are known to be produced as a result of intravesical BCG therapy for bladder cancer (IFNγ, TNFα, IL-1) are able to regulate the expression of several molecules which are important for a wide variety of immunological interactions. When recombinant human IFNγ or TNFα are applied to bladder tumour cell lines *in vitro* the expression of ICAM-1 and HLA class II molecules is induced or augmented.

These studies provide interesting and useful data on the disease of bladder cancer, the possible mechanisms of successful response to immunotherapy, and the mode of action of lymphokine-activated killer cells. However, if the molecules discussed are not expressed by bladder cancer cells, if the cytokines thought to be produced as a direct result of BCG therapy do not exert immunomodulatory and cytotoxic effects on bladder cancer cells, and if the lymphocytes which infiltrate the bladder wall do not interact with the tumour cells, then these *in vitro* studies are of limited use. For these reasons it was thought vital that some reference be made to the clinical situation of bladder cancer and its immunotherapy.

3.6.1 The effect of urine from patients receiving intravesical BCG therapy

The work of several major urological research groups has demonstrated the existence of a variety of cytokines in the urine of patients who had received intravesical BCG for the treatment and prophylaxis of superficial bladder cancer. The effects of these cytokines have been investigated by stimulating bladder cancer cells *in vitro* with purified, recombinant human cytokines, either as single agents or in combination with one other cytokine. Although this provides useful information on the possible role of such molecules, these studies are limited by the very purity of the cytokines used. In urine, a vast and complex "cocktail" of cytokines is produced by patients who respond to BCG therapy. In addition, other non-cytokine, immunologically relevant molecules such as soluble receptors are known to be secreted in urine. The effect of such urine *per se*, however, has not been investigated. The aims of this area of work were to determine if BCG urine, and the mixture of molecules contained within, were able to mimic any of the findings *in vitro.*

From the work of Prescott *et al* (1989), it would seem that following repeated BCG instillation in to the bladder cytokines are produced during the twelve hours post treatment. For this reason, the following experiments were conducted using urine obtained during the twelve hours following instillation. Urine from each of the six therapeutic instillations was collected and processed prior to storage. There exists some controversy concerning the presence of IFNγ in the urine of bladder cancer patients. One possible reason for this is the acid labile nature of this cytokine. Even short term exposure to a pH of 6.5 can cause a loss of biological activity. It was decided, therefore, to dialyze the urine against PBS (pH7.2) in order to prevent such destruction of cytokines taking

place.

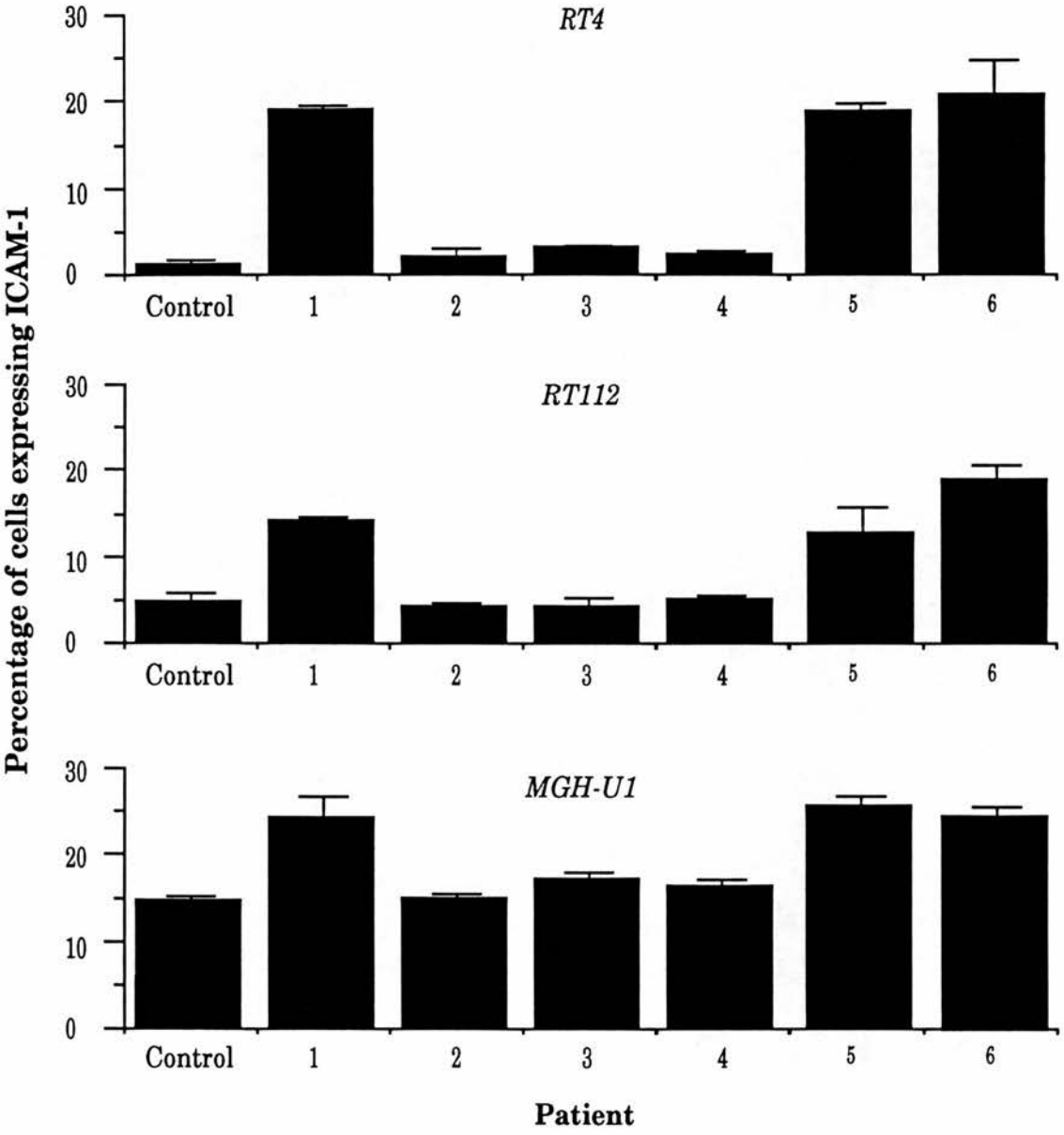
Bladder cancer cell lines were stimulated with the dialyzed urine collected over the twelve hours following each instillation of BCG. The expression of both ICAM-1 and HLA class II molecules was monitored using flow cytometry; ICAM-1 expression was determined after 24 hours and HLA class II expression after 48 hours of stimulation.

There was a heterogeneous response to the urine from BCG treated patients. When the cell lines RT4, RT112 and MGH-U1 were stimulated with urine taken from the first instillation, three urines failed to induce or augment ICAM-1 expression, however, the urine of three patients caused an increase in antigen expression (Figure 75). When cells were stimulated for 24 hours with instillation 6 urine, a significant ($p<0.01$) increase in ICAM-1 expression was observed (Figure 76). The effects were of similar magnitude on each of the three cell lines. The increase was not identical for all the patient samples tested. Urine from patient 3 only stimulated a small increase in ICAM-1 expression when compared to patients, 1, 2 or 4, whilst patients 5 and 6 gave an intermediate response.

The immunomodulatory effects of urine from the sixth instillation of BCG were apparent at dilutions as high as 1:32 (Figure 77). The biological effects of this urine were equivalent to $100Uml^{-1}$ of recombinant IFNγ (if due to IFNγ alone).

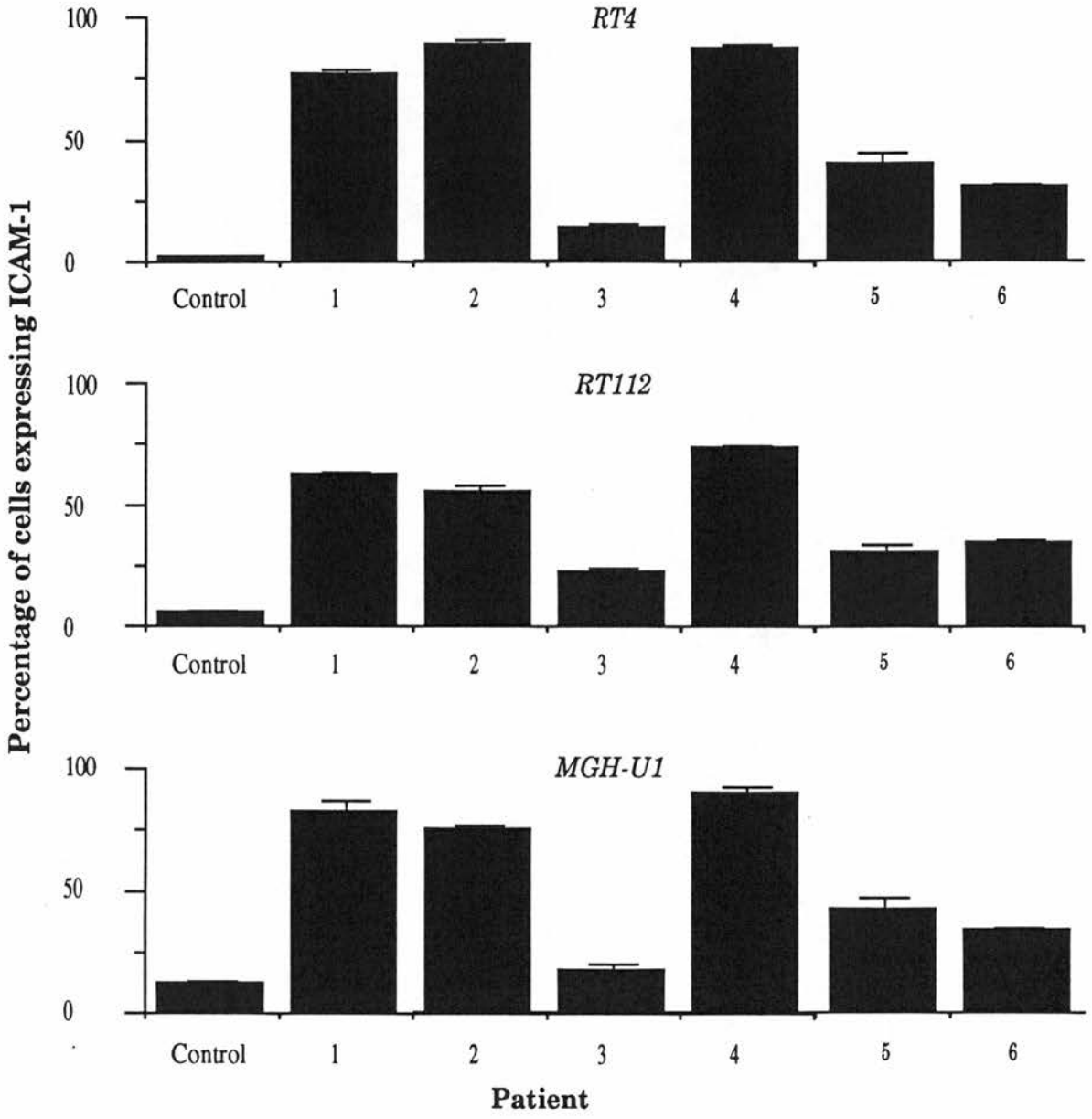
The effect of urine from each of the six instillations on both ICAM-1 and HLA class II molecule expression was investigated (Figures 78 and 79). Urine from earlier instillations (1 and 2) generally failed to elicit a response. Urine from the latter instillations induced the expression of ICAM-1 and HLA class II antigens. Generally, the maximal response was observed with samples obtained following the latter instillations, however, the responses were heterogeneous. One point of interest may be observed

Figure 75. The effect of instillation one urine on ICAM-1 expression



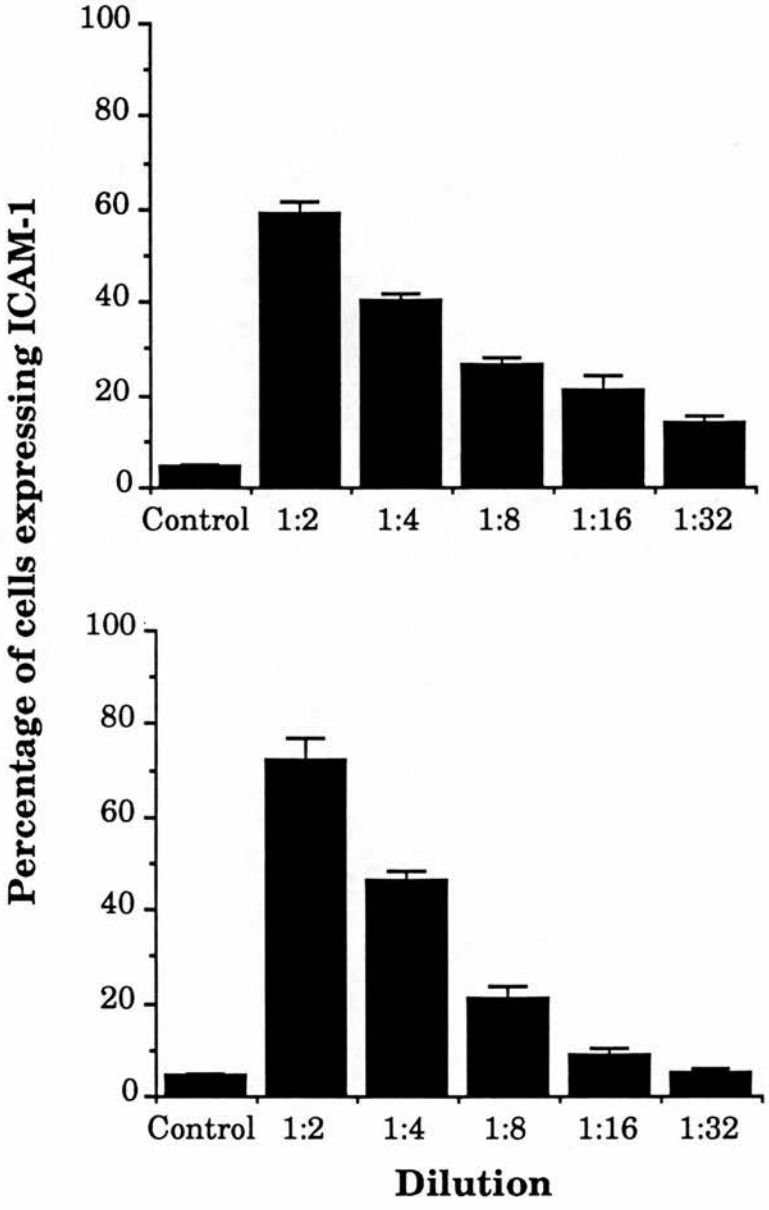
Cells were stimulated for 24 hours with dialyzed urine (pH7.2) which had been diluted 1:2 in complete medium. The expression of ICAM-1 was determined using flowcytometry with the monoclonal antibody RR1/1. Bars represent the mean of triplicate determinations, error bars represent 1 standard deviation.

Fig. 76 The effect of instillation six urine on ICAM-1 expression



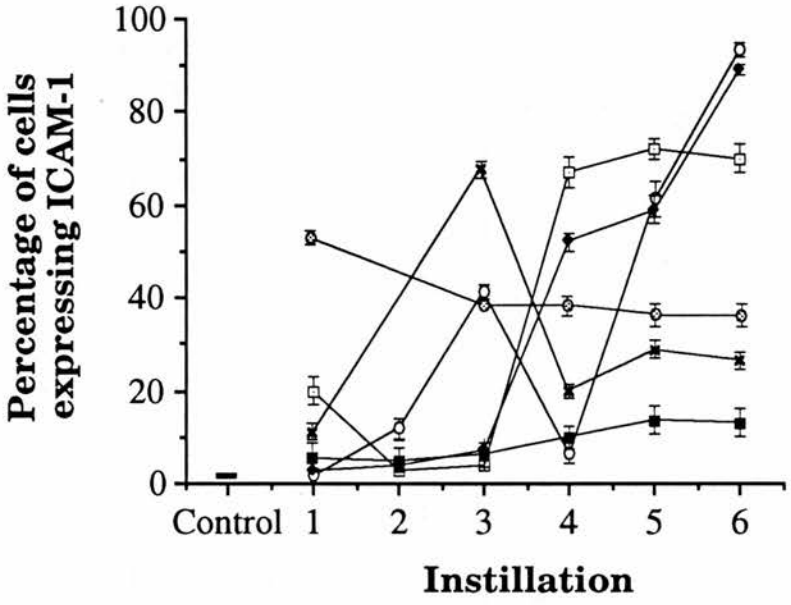
Cells were stimulated for 24 hours with dialyzed urine (pH7.2) which had been diluted 1:2 in complete medium. The expression of ICAM-1 was determined using flowcytometry with the monoclonal antibody RR1/1. Bars represent the mean of triplicate determinations, error bars represent 1 standard deviation.

Fig 77. Concentration dependent effect of urine on ICAM-1 expression



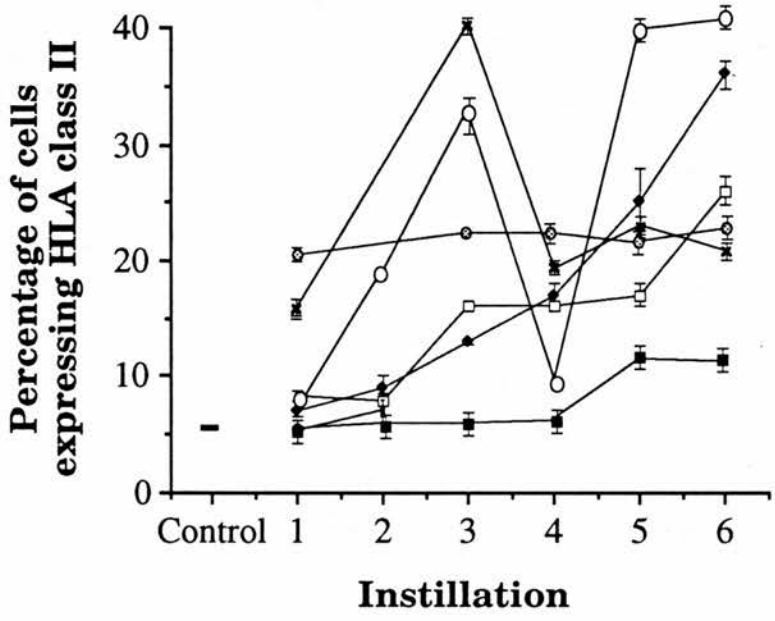
The effect of BCG urine from the sixth instillation on the expression of ICAM-1 by the cell line RT4 (G1). Dialyzed urine was diluted in complete medium and the cells incubated in its presence for 24 hours. The expression of ICAM-1 was determined via flow cytometry using monoclonal antibody probes. Bars represent the mean of triplicate determinations and error bars represent 1 standard deviation.

Fig 78. The induction of ICAM-1 expression by urine from each of six instillations



The biological response of the RT4 bladder cancer cell lines to urine collected during the first twelve hours following each of six instillations of BCG. Urine was first dialyzed and then added to the cells for 24 hours at a dilution of 1:2. The urine from six patients was analyzed; patient 1 (—□—), patient 2 (—◆—), patient 3 (—■—), patient 4 (—○—), patient 5 (—⊙—), and patient six (—✕—). The expression of ICAM-1 is shown and was determined by flow cytometry with the monoclonal antibody RR1/1.

Fig 79. The induction of MHC class II expression by urine from each of six instillations



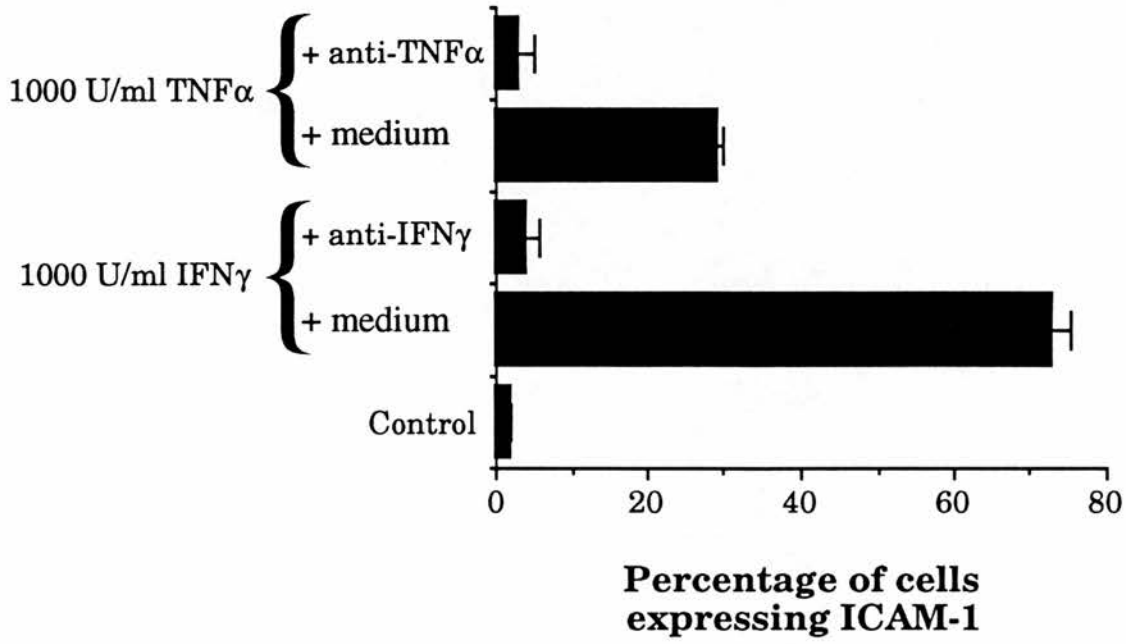
The biological response of the RT4 bladder cancer cell lines to urine collected during the first twelve hours following each of six instillations of BCG. Urine was first dialyzed and then added to the cells for 24 hours at a dilution of 1:2. The urine from six patients was analyzed; patient 1 (—□—), patient 2 (—●—), patient 3 (—■—), patient 4 (—○—), patient 5 (—◎—), and patient six (—✖—). The expression of HLA-class II was determined by flow cytometry with the monoclonal antibody DA6-231.

with patient 4 urine, instillation 5. At this point the biological response to urine is poor and not greatly raised above the control values. Retrospective enquiries revealed that the fifth instillation had not been given to that particular patient due to a prolonged reaction to earlier instillations. However, a urine sample was still collected by the patient and this was processed as usual.

3.8.2 Controls for neutralizing experiments

One approach commonly used to dissect out the role of specific cytokines in a complex mixture of substances is to use antibodies which not only specifically bind to the cytokine, but also neutralize its biological effects. Such neutralizing antibodies are often polyclonal in nature and are therefore extremely effective at inhibiting the biological response. Bladder cancer cells were stimulated with recombinant IFNγ and TNFα for 24 hours in the presence or absence of neutralizing antibodies to both these cytokines. When neutralizing antibodies were used at a 1:100 dilution they totally abolished the effect of these cytokines on ICAM-1 expression (Figure 80). When RT4 cells were stimulated with 1,000Uml^{-1} of either IFNγ or TNFα a large biological response was seen, as expected. In the presence of a 1:100 dilution of antibody this effect was completely negated. It should be noted that 1000Uml^{-1} of both cytokines corresponds to a level far greater than that detected in the urine following BCG instillation. This concentration of cytokine was chosen as it is approximately one order greater than that found in urine.

Fig 80. Controls for neutralizing experiments



Neutralizing antibodies to IFNγ and TNFα can effectively negate the biological effect of these cytokines. Cells were stimulated with 1,000U/ml of either cytokine in the presence and absence of a 1:100 dilution of neutralizing polyclonal antibody specific for that cytokine. Following a 24 hour incubation the percentage of cells expressing ICAM-1 on their surface was determined using flow cytometry. Solid represent the mean of triplicate determinations, error bars indicate 1 standard deviation.

3.8.3 Neutralizing antibodies to IFNγ but not TNFα abolish the immunomodulatory effects of BCG urine

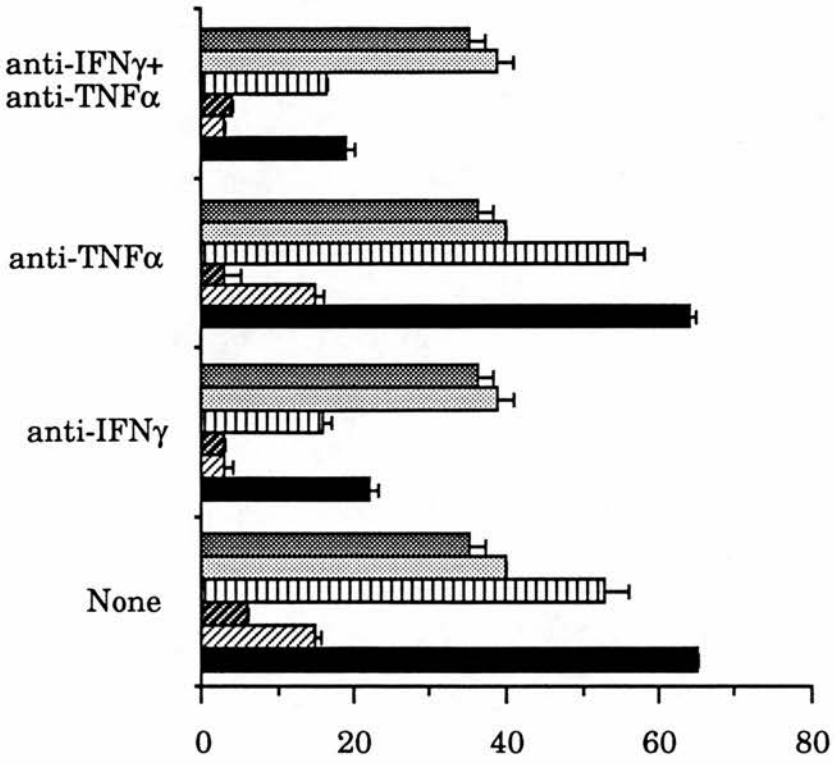
When urine from sixth instillation samples was pre-incubated with neutralizing antibody to IFNγ, its ability to modulate ICAM-1 expression was almost completely negated ($p<0.01$) (Figure 81). However, some activity remained un-neutralized by anti-IFNγ antibodies. When samples were pre-incubated with neutralizing antibodies to TNFα, no significant decrease in ICAM-1 expression was observed for any patient. Simultaneous pre-incubation with both antibodies had no greater effect than with anti-IFNγ antibodies alone.

3.8.4 Detection of IFNγ in the urine following BCG therapy

Although the above biological assay with neutralizing antibodies demonstrated the presence of IFNγ in the urine of patients who had received intravesical BCG vaccine, it was decided to use immunological methods in order to confirm its existence. As previously mentioned considerable controversy exists over the presence of this cytokine in the urine of patients receiving such adjuvant immunotherapy (E. de Boer and A. Böhle, personal communications). For this reason two independent commercially available immunoassay kits were employed to quantify the IFNγ in the urine samples.

No difference in the concentration of IFNγ detected in the urine was evident when two different immunoassay systems were employed (data not shown). Using the two site ELISA assay and an immunoradiometric assay, the presence of IFNγ in urine was confirmed. Peak levels as high as 100 Uml^{-1} of urine were detected in the latter samples. If the volume of

Fig 81. Antibodies to IFNγ abolish the immunomodulatory effects of BCG urine



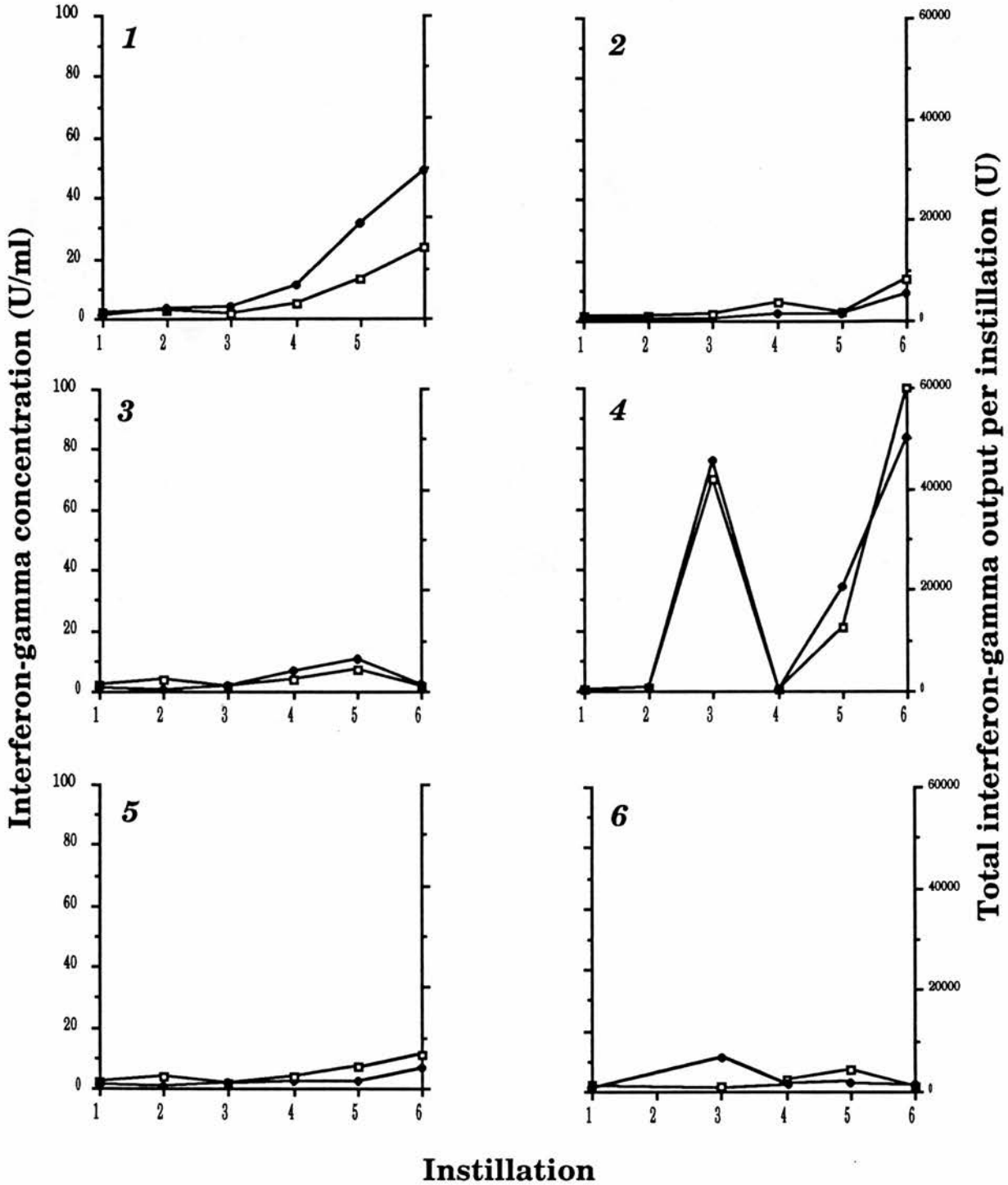
The effect of neutralizing polyclonal antibodies to IFNγ and/or TNFα on the immunomodulatory potential of urine taken from patients receiving BCG therapy. Urine from the twelve hours following instillation six was dialyzed against PBS (pH7.2). Samples for neutralization experiments were incubated with a 1:100 dilution of polyclonal antibody for 30 minutes at 20C prior to stimulating RT4 cells for 24 hours. Bars represent the mean of triplicate determinations. Error bars show 1 standard deviation. Six patients were used; #1 (■), #2 (▨), #3 (▨), #4 (▥), #5 (▤) and #6 (▩).

urine produced during the twelve hours post instillation is considered then total IFNγ production can be assessed. Figure 82 shows the concentration of IFNγ in the urine and the total IFNγ production during the first twelve hours following the sixth instillation of BCG. It should also be noted that the concentration of IFNγ in the urine of patients 2, 3, 5 and 6 never exceeded 15 Uml^{-1} whilst the other patients secreted higher levels. Patient 4 secreted levels in excess of 100 Uml^{-1}. The total IFNγ production per instillation varied with each patient. Patients 1 and 4 produced peak levels in excess of 30,000U per instillation. However, no change in the ranking of the patients was evident when urine volume (*ie* total output) was taken into consideration.

3.8.5 Detection of TNFα in the urine following BCG therapy

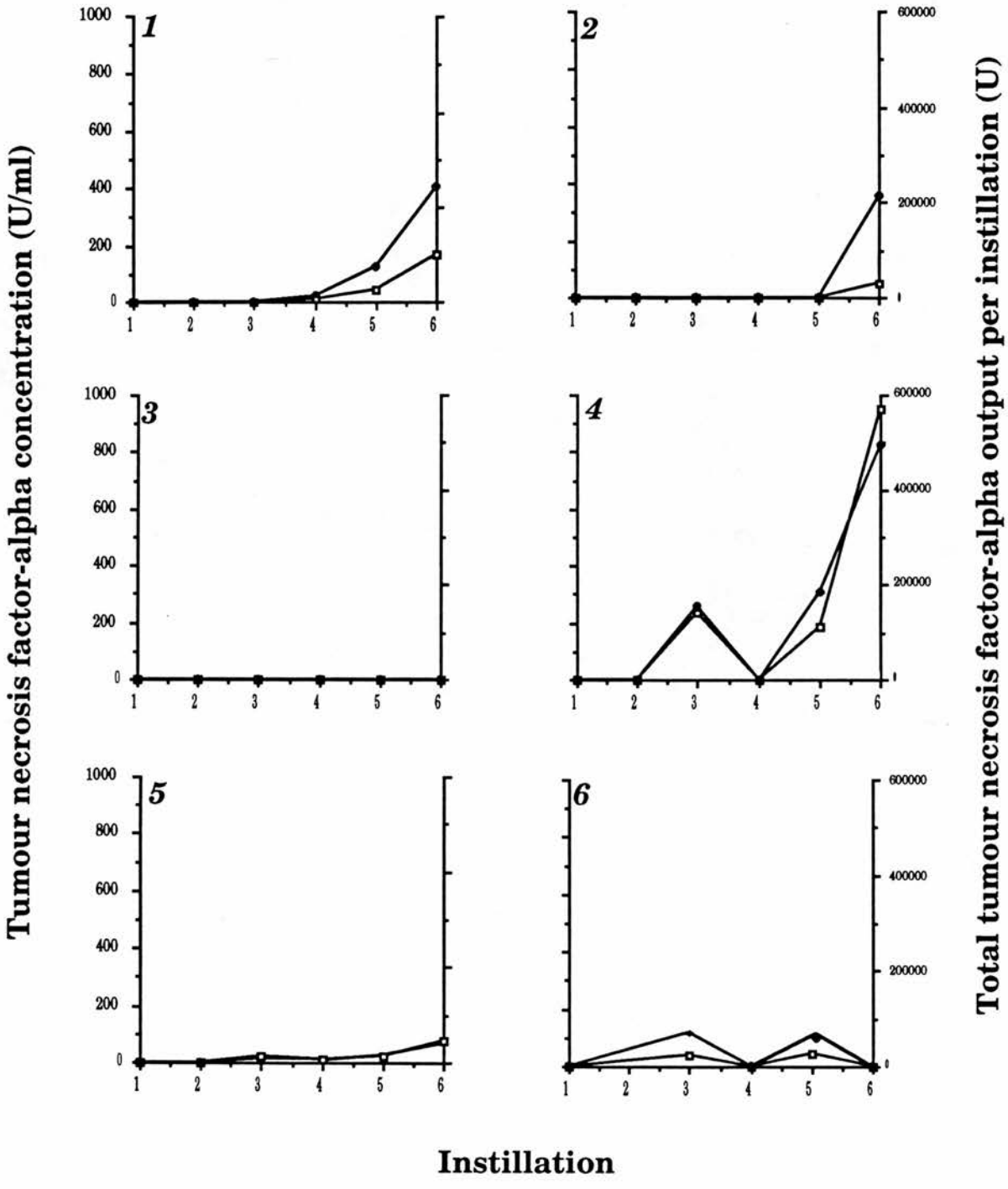
The work of Böhle and colleagues (1990) has demonstrated the presence of TNFα in patient's urine following BCG therapy. Although TNFα did not appear to have an important role in the biological assay of urine, its presence was non-the-less investigated. Using a commercially available ELISA kit the quantity of TNFα in the urine was determined (Figure 83). Not all patients produced detectable levels of TNFα in their urine. Patient 3 failed to produce any. However, as with the production of IFNγ, a vast heterogeneity of response was observed. There was general agreement between the production of IFNγ and the production of TNFα. Again patient 4 produced the highest levels, with peak production occurring following the sixth instillation (>900Uml-1). Upon consideration of the urine volume, patient 4 maximally produced nearly $6x10^5$U/instillation.

Fig 82. The secretion of IFNγ into the urine of patients receiving BCG therapy



The production of IFNγ and its subsequent secretion into the urine of patients receiving intravesical BCG therapy. Urine was collected from the twelve hours following each of six instillations of BCG. Dialyzed urine (pH7.2) was then assayed for IFNγ using an ELISA kit system. Shown is the concentration of interferon (—□—), and the total output per instillation (—●—) for each of six patients tested.

Fig 83. The secretion of TNFα into the urine of patients receiving BCG therapy



The production of TNFα and its subsequent secretion into the urine of patients receiving intravesical BCG therapy. Urine was collected from the twelve hours following each of six instillations of BCG. Dialyzed urine (pH7.2) was then assayed for TNFα using an ELISA kit system. Shown is the concentration of interferon (—□—), and the total output per instillation (—●—) for each of six patients tested.

3.8.6 The response of L929 murine fibroblasts to urine containing TNFα

Immunoassay detection systems have identified the presence of high levels of TNFα in the urine of patients following BCG therapy, however, three bladder cancer cell lines apparently failed to respond to such TNF, thereby questioning the biological capabilities of such TNF. It was decided that the TNF sensitive L929 bioassay should be used to further investigate such a possibility. The urine from the sixth instillation of four patients was applied to the L929 system, as were the relevant controls and standards. Although recombinant TNF proved toxic to the L929 cells, the urine from all four patients was not found to exert any significant cytotoxicity (data not shown).

3.8.7 The secretion of soluble ICAM-1 molecules into the urine following intravesical BCG therapy

In recent years several forms of non-cytokine, immunologically relevant soluble molecules have been identified, often in the sera of normal individuals or in that of patients with malignancies or chronic pathological states (Lisoni *et al* 1989). A form of circulating ICAM-1 (cICAM-1) has been identified in the sera of normal humans and the the sera of patients with leucocyte adhesion deficiency (LAD) (Rothlein *et al* 1991). Levels of cICAM-1 in normal individuals were around 100-200ngml^{-1}. In individuals with LAD elevated levels of cICAM-1 were detected (200-700ngml^{-1}). *In vitro,* both cICAM-1 and sICAM-1 have been shown to be fully functional in that they mediate LFA-1 dependent lymphocyte adhesion. The cellular source of cICAM-1 was deemed to be from mononuclear cells. The authors proposed that cICAM-1 may act as a useful marker for inflammatory

disease. Elevated cICAM-1 expression has also been correlated with a number of malignant diseases (Tsujisaka *et al* 1991). An increased propensity to metastasise in malignant melanoma has been correlated with an increase in the expression of membrane associated ICAM-1.

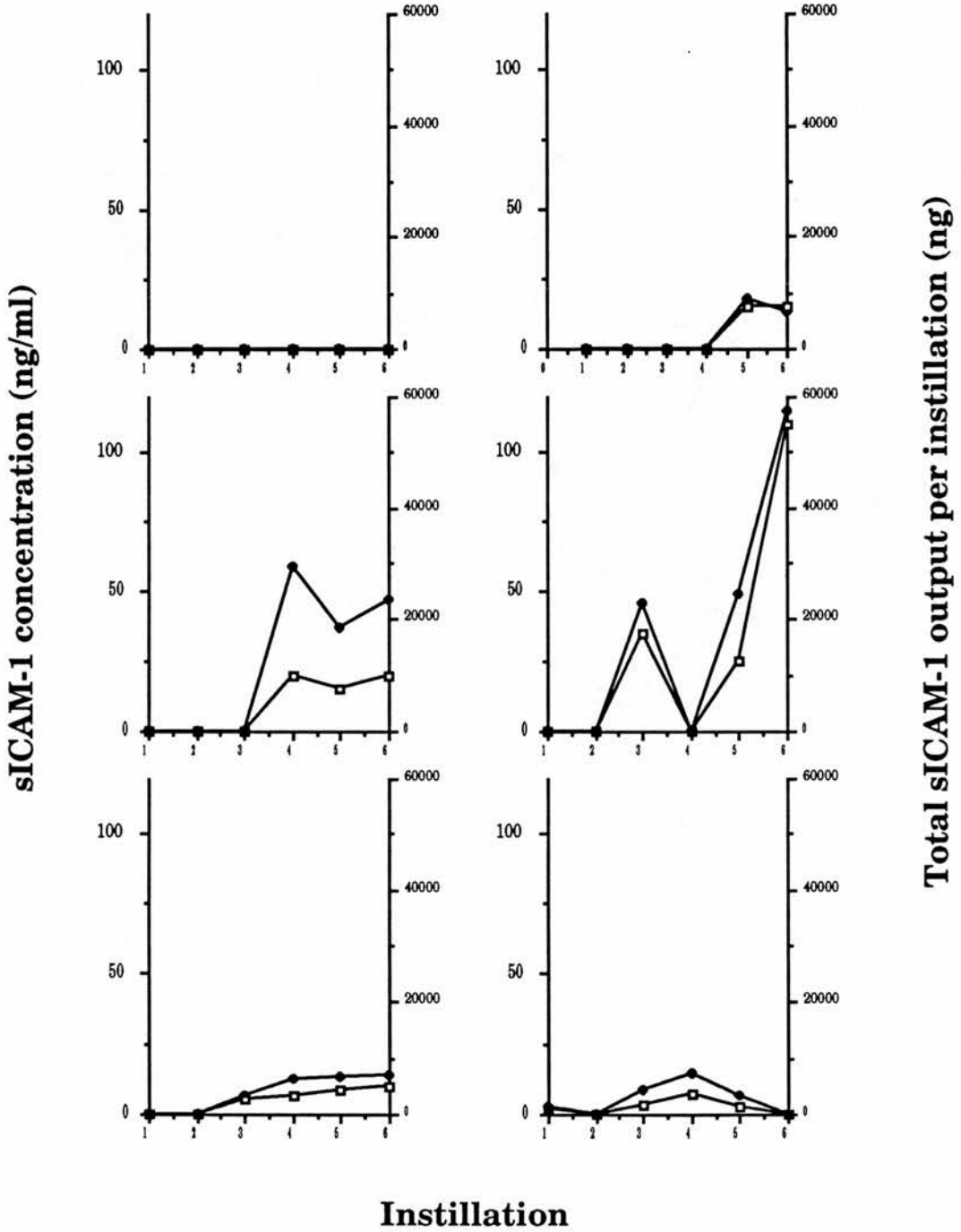
Using a sandwich ELISA assay system we studied the secretion of sICAM-1 into the urine following intravesical BCG administration. The presence of soluble ICAM-1 was not detected in the urine of all patients (Figure 84). Patient 1, in whose urine large amounts of both IFNγ and TNFα had been detected, failed to secrete any ICAM-1 into the urine. In contrast, patient 3 who produced relatively low levels of IFNγ, secreted detectable levels of sICAM-1. Some patients who produced moderate or high levels of IFNγ also secreted moderate or high levels of sICAM-1 (patients 2 and 4). The maximum concentration of sICAM-1 detected in the urine was observed in patient 4 following the sixth instillation ($120ngml^{-1}$).

When the urine volume was considered the maximal level of sICAM-1 was equivalent to nearly 6μg per instillation. This level of sICAM-1 is equivalent to $3.7x10^{13}$ molecules per instillation. Assuming the same rate of production per cell as was demonstrated for the SD cell line, this would correspond to secretion by $1.7x10^{8}$ cells.

3.8.8 The expression of ICAM-1 and ICAM-2 by bladder tumours

Several tumour types have been found to express the adhesion molecule ICAM-1, including malignant melanoma and colonic carcinoma. The constitutive expression of ICAM-1 and ICAM-2 by bladder cancer cell lines has been demonstrated, however, *in vivo*, no information exists as to the expression of ICAM-1 and ICAM-2 by tumour cells. The constitutive

Fig 84. The secretion of sICAM-1 into the urine of patients receiving BCG therapy



The production of sICAM-1 and subsequent secretion into the urine of patients receiving intravesical BCG therapy. Urine was collected from the twelve hours following each of six instillations of BCG. Dialyzed urine (pH7.2) was then assayed for ICAM-1 using an ELISA kit system. Shown is the concentration of sICAM-1 (—□—), and the total output per instillation (—●—) for each of six patients tested.

expression of ICAM molecules by bladder tumour was investigated using 5μm frozen section obtained from tissue removed by cystoscopic resection of tumour. Tumours representing various grades and stages of bladder cancer were investigated. The results are summarized in Table 9. The majority of tumour cells failed to express ICAM-1. A small percentage of T1 and T2 tumours expressed ICAM-1. Even fewer tumours expressed ICAM-2. Isolated positive events were recorded in some tumours but these were thought to be macrophages. The stromal tissue located between areas of tumour was often strongly positive for ICAM-1, and sometimes positive for ICAM-2. The vascular endothelium of small vessels throughout the section were strongly stained for ICAM-1 and ICAM-2 (data not shown). In Plates 6 and 7, tandem sections of a superficial bladder tumour are stained with antibodies to ICAM-1 and ICAM-2. No staining was identified in the tumour, only in the surrounding stroma.

3.8.9 The expression of ICAM-1 by freshly derived bladder tumour cells

The expression of ICAM-1 by bladder tumour cells was studied by flow cytometry. A unicellular suspension of tumour cells was prepared from freshly ressected material which had been purified by density centrifugation. The tumours of six patients of various grades were studied by this method. Few or no cells were found to express ICAM-1 (data not shown). The maximum percentage of cells found to express ICAM-1 was 3.5%. This result confirms the above immunohistochemical observations and findings in that bladder tumour cells do not constitutively express the ICAM-1 molecule.

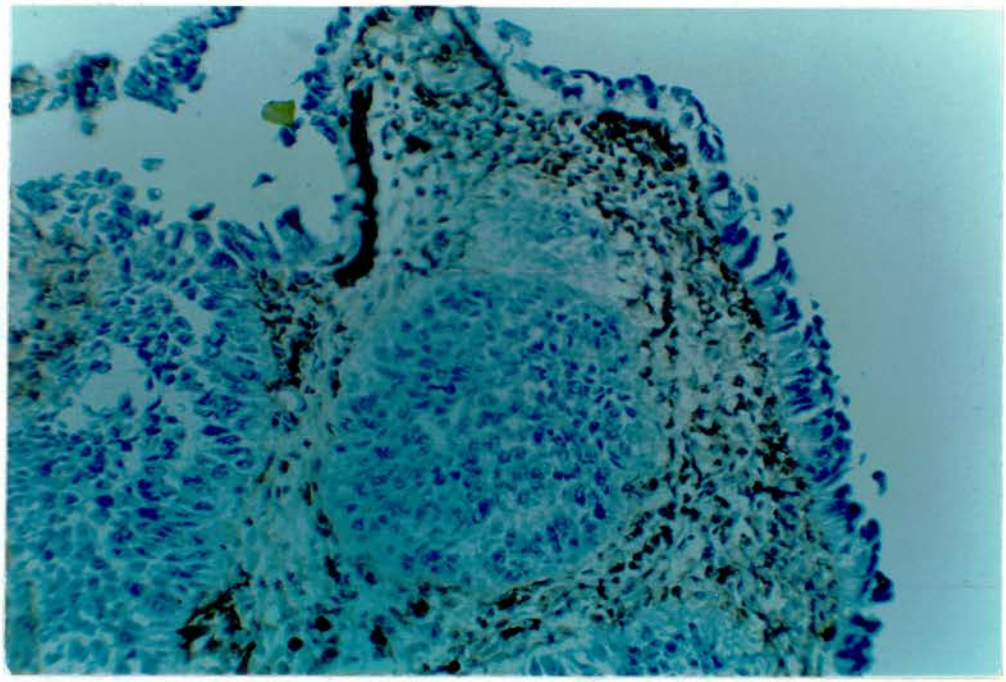
Table 9. The expression of ICAM-1 and -2 by bladder tumours

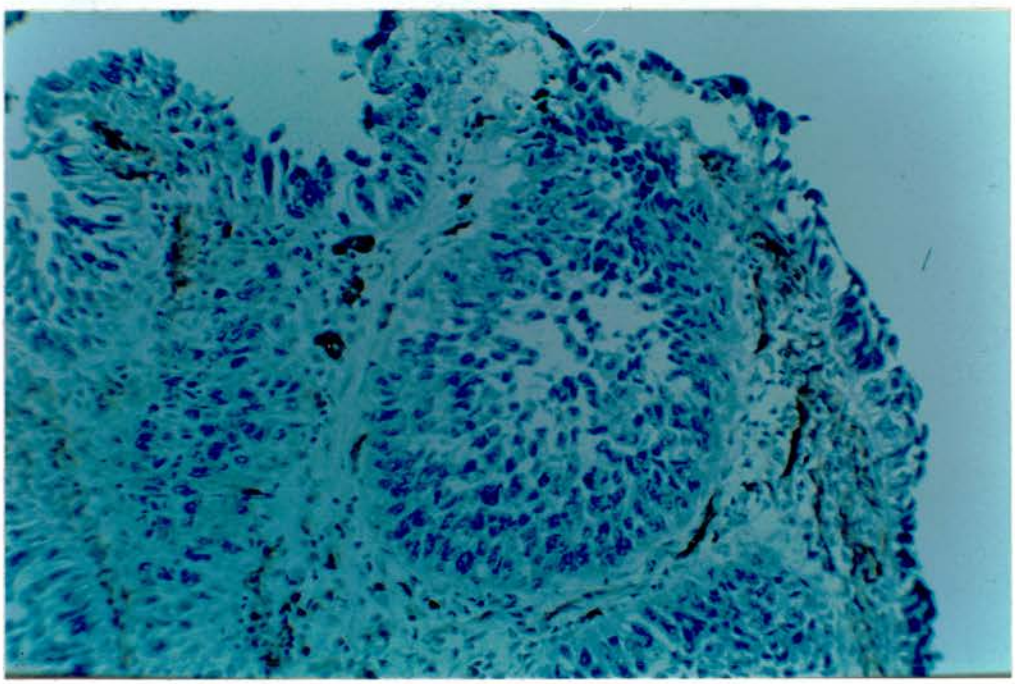
		Tumour							
		ICAM-1 (%)				ICAM-2 %			
Stage	Mean Grade	++	+	+/-	-	++	+	+/-	-
Ta	1.6	0	0	25	75	0	0	0	100
T1	2.5	25	25	25	25	0	25	0	75
T2	3.0	20	0	75	25	0	0	20	80
T3+	3.0	0	10	28	58	14	0	0	86

		Stroma							
		ICAM-1 (%)				ICAM-2 %			
Stage	Mean Grade	++	+	+/-	-	++	+	+/-	-
Ta	1.6	60	0	40	0	0	0	80	20
T1	2.5	75	0	25	0	25	50	0	25
T2	3.0	60	0	20	20	20	0	40	40
T3+	3.0	67	0	0	33	32	0	32	36

The expression of ICAM-1 and ICAM-2 by bladder tumour and the surrounding stromal tissue. Frozen sections were immunohistochemically stained for either ICAM-1 or ICAM-2. The sections were scored as follows: 0% of cells expressing (-), 0-5% of cells expressing (+/-), 5-30% of cells expressing (+), >30% of cells expressing. The results are shown ordered for tumour stage and the mean grade for each stage is also shown.

Plates 6 and 7.





The immunohistochemical localization of ICAM-1 (upper) and ICAM-2 (lower) in tandem frozen sections of a high grade superficial bladder tumour. The expression of the ICAM genes is restricted to the stroma whilst the tumour remains negative.

4 Discussion

Bladder cancer is a common disease with increasing incidence and mortality in the western world (Koroltchouk *et al* 1987). Fortunately the majority of patients present with superficial disease which is not immediately life threatening. Unfortunately, a significant proportion of patients will progress to invasive disease, associated with which is a high degree of mortality. In the United States of America almost all patients who present with superficial disease will receive intravesical adjuvant immunotherapy consisting of repeated instillations of viable BCG vaccine (D. Lamm, personal communication). In the United Kingdom, however, many patients still receive conventional therapy consisting of surgical resection, chemotherapy and radiotherapy.

In these studies, a series of human tumour cell lines representing different histopathological grades of superficial bladder cancer was used as a model for the analysis of the possible mechanisms of immunotherapy as employed in the treatment of such tumours: the histopathological grades and growth characteristics of these cell lines correspond to those of the parent tumour of similar grade.

4.1 The sensitivity of bladder cancer cells to natural and cytokine enhanced cellular cytotoxicity

A limited variety of tumour cell lines and freshly isolated tumour cells are susceptible to killing by NK cells (Serrate *et al* 1982). Such cells *in vivo* may be more easily destroyed by the host than NK resistant tumour cells. Unfortunately, the majority of tumour cells would appear to fall into this latter category, therefore presenting the host with increased

difficulties. A series of major research initiatives have been directed towards the identification of tumour specific antigens (Baldwin 1966. Lancki *et al* 1991). Several virally induced tumours have been demonstrated to express such molecules, however it would seem that most tumours do not (Schreiber *et al* 1988). Some tumours express tumour associated antigens such as CEA and AFP, however these have proved of limited use in anti-tumour strategies. The discovery of a novel killing capability, distinct from T-cell and NK mediated cytotoxicity was heralded as a breakthrough for cancer therapy (Grimm *et al* 1982). However, with only two exceptions (namely renal cell carcinoma and malignant melanoma) the clinical use of LAK cells has proved disappointing.

The preliminary experiments for this series of investigations showed that human bladder cancer cells were not killed by the NK activity resident in normal peripheral blood. However, following activation with recombinant IL-2 the exhibition of LAK activity against all eight bladder cancer cell lines was observed. The kinetics and IL-2 requirements of LAK generation were similar to those reported by other workers; namely optimal LAK activity was observed after six days of culture with 1,000Uml^{-1} of IL-2 (Grimm *et al* 1982).

It is well known that cytokines other than IL-2 partake in the generation of LAK activity (Kaufmann *et al* 1991. Matossian-Rogers *et al* 1989). Furthermore, other cytokines when used as the primary stimuli can generate LAK activity (Matossian-Rogers *et al* 1989). In these studies both TNFα and IFNα were found to marginally stimulate the generation of LAK activity. The levels of cytotoxicity achieved with these stimuli were lower than achieved with IL-2 alone. When placed in combination with IL-2, both IFNα and TNFα were seen to behave synergistically in that LAK activity was increased to levels above that

achieved with either agent alone. If LAK cell therapy was to be employed then a fundamental requirement would be maximal cytotoxic potential, in such a case several cytokines might be used to achieve this.

Of interest was the differential susceptibility of the tumour cell lines to LAK activity. As there was little difference in LAK activity generated from different donors and as the ranking of susceptibility was consistent, the differential susceptibility was not considered to be attributable to the effector cell. There was no evidence of correlation between tumour grade and LAK susceptibility.

When freshly isolated tumour cells were mixed with LAK cells they were killed, all be it in a differential manner. It is of interest that recent clinical trials with LAK cells for the treatment of bladder cancer have been completed (Hermann *et al* 1992). When LAK cells were administered systemically no clinical responses were observed despite changes in several pertinent immunological parameters, namely increase in leucocytes, activation of NK cells and infiltration of the tumour with activated lymphocytes. Whether such cells if administered intralesional or adjacent to the tumour would give a clinical response remains to be investigated. However, the bladder is easily accessible and intralesional administration of LAK cells may be more effective for the treatment of advanced, muscle invasive bladder cancer. Furthermore, local administration of IL-2/LAK cells would have considerably lower cytotoxicity than that observed with systemic administration.

4.2 The expression and modulation of adhesion molecules

From the early studies it is apparent that the differential susceptibility of bladder tumour cells would appear to be attributable to

differences in the target cells rather than to the effector cells. Therefore, investigations were undertaken to dissect mechanisms which would account for such differences.

Scanning electron micrography of the interaction between effector and target cells revealed the adhesion of effector to target cells. Such adhesion appeared to be strong as the separation of two cells after adhesion did not occur at the points of adhesion but through the cell body. For these reasons a phenotypic evaluation of adhesion molecules expression by bladder cancer cells was undertaken.

The expression of HLA class I molecules by bladder cancer cell lines is ubiquitous, a fact which could account for the insensitivity of such lines to NK activity, as a negative correlation has been established between HLA class I expression and NK mediated killing (Moretta *et al* 1992). In particular the loss of one particular HLA class I allele, Cw3, causes cells to become susceptible to NK lysis. Interestingly, the RT4 cell line constitutively expressed low levels of HLA class II molecules on its surface. One of the most striking differences between these closely related groups of molecules is their tissue distribution. The MHC class I antigens are widely distributed, being expressed by most nucleated cell types in varying amounts. The MHC class II antigens have a highly restricted tissue distribution: they are found primarily on certain cells of the immune system, and can be induced on a few other cell types. The restricted expression of class II antigens is closely related to their function; indeed the maintenance of tissue specific expression appears to be critical for normal function of the immune system. The adhesion molecule NCAM is thought to be an important ligand for NK activity, mediating homophillic adhesion between NK and target cell, however it presence was not confirmed on bladder cancer cells. Neither was VCAM, the ligand for VLA-

4. Such molecules were candidates for the interaction between LAK and target cells. The existence of two members of the ICAM family of adhesion molecules on bladder cancer cells has been established. Although no significant conclusions can be drawn, the expression was indicative of a relationship to the tumour grade. Low grade cells express ICAM-2, a molecule not identified on the G2 and G3 cell lines. The higher grade cells constitutively express ICAM-1 at varying levels. The significance of a grade related expression remains undetermined. However, several reports have suggested that ICAM-1 expression correlates with an increased propensity to metastasize (Tsujisaka *et al* 1991). It is established that high grade bladder tumours have a worse prognosis than low grade tumours (Heney *et al* 1983).

Following the administration of intravesical BCG a variety of cytokines are produced and can be detected in the patients urine (Böhle *et al* 1990. Prescott *et al* 1989. Fleischmann *et al* 1989). The effects of these cytokines on bladder cancer cells has been investigated in order to investigate their possible anti-tumour mechanisms. Interferon-gamma is a potent modulator of ICAM-1 expression on bladder cancer cell lines. Following stimulation with IFNγ ICAM-1 expression is rapidly induced. On vascular endothelium such rapid induction of ICAM-1 expression serves an obvious function, that of an anchorage point for leucocytes prior to their transmigration to sites of trauma (Figure 4). However, the role of ICAM-1 expression on bladder cancer cells is not so blatant. Hawkyard and colleagues (1991 and 1992) have demonstrated other actions of IFNγ on bladder cancer cells. *In vitro,* IFNγ induced the expression of MHC class II molecules on three bladder cancer cell lines in a grade dependent manner (G1 - highly inducible, G3 - poorly responsive). However, unlike ICAM-1, the induction of class II required high concentrations of IFNγ (1,000Uml^{-1})

and prolonged exposure (>100 hours in some cases). One possible explanation for this may lie in the reported down-regulation of MHC genes due to activated oncogenes and their products (Morris 1990) the implications of which remein to be determined. Hawkyard *et al* also demonstrated a grade related cytotoxic and cytostatic effect directly exerted by IFNγ on bladder cancer cells (Hawkyard *et al* 1992). These effects, like those involving class II expression, also required several days exposure to high concentrations of cytokine. It would appear that bladder cancer cells respond in a variety of ways following exposure to recombinant IFNγ. The initial response is increased ICAM-1 expression, found to be maximal after 24 hours of continuous stimulation. Secondly, following a further 24-72 hours the tumour cells express MHC class II molecules. Thirdly, continuous exposure to IFNγ results in cell death, or at very least an arrest of cell proliferation. The mechanisms of such scheduled events remain undelineated. Although VCAM is a cytokine inducible adhesion molecule, its expression was not increased following stimulation with IFNγ. Furthermore, the expression of NCAM and ICAM-2 remain unaltered following similar stimulation.

Cytokines other than IFNγ are produced in response to BCG therapy, these include IL-1, IL-2 and TNFα. An increase in ICAM-1 expression is mediated by stimulation with TNFα and IL-1α. In general, the effect of these cytokines is less than IFNγ mediated effects. However, in combination with IFNγ, TNFα acts synergistically to increase the level of ICAM-1 expression. As cytokines are probably never produced singly, the combined results of several cytokines are of greater importance than those of a single agent. Bladder cancer cells do not respond with modified ICAM-1 expression to a variety of other cytokines, some of which are detected in the urine (*eg* IL-2) and some not (*eg* IL-4, K. James personal

communication). These cytokines also do not modify MHC class II expression by similar cells (Hawkyard, personal communication). Therefore, the role of such cytokines in the response to BCG may not directly involve the tumour cell, but instead may severe as activation signals to infiltrating lymphocytes, NK cells, neutrophils and macrophages.

Bladder cancer cells respond to IFNγ in a heterogeneous manner. Cells which respond poorly in terms of class II expression (*eg* MGH-U1) may respond well in terms of ICAM-1 expression, and *vice versa*. One reason for this may be that MGH-U1 does not constitutively express MHC class II but does express ICAM-1. Therefore, the gene for ICAM-1 is already partially activated. Interestingly, recent evidence from our laboratory has shown that MGH-U1 cells constitutively secrete TNFα (A. Alexandrov personal communication) a factor which may account for the constitutive expression of ICAM-1 by this cell line. The nature of the response is dependent upon the concentration of cytokine. Therefore, with $100Uml^{-1}$ IFNγ, MGH-U1 will express elevated levels of ICAM-1 but will not express MHC class II antigens, with $1000Uml^{-1}$ both ICAM-1 and MHC class II antigens will be expressed. Therefore, it is possible to gain insight into how different concentrations of cytokine may exert different effects on their target. In classical T-cell communication both MHC antigens and ICAM-1 are required (Altmann *et al* 1989), however, less specific cytotoxic mechanisms may only require the expression of ICAM-1 for the necessary communication to occur. Most recently, Lattime and colleagues (1992) have investigated the antigen presenting ability of murine bladder cancer cells. These cells, which usually fail to express MHC class II antigens, express class II molecules following stimulation with IFNγ ($100Uml^{-1}$). Furthermore, their most alluring finding was that

these tumour cells presented BCG to specific CD4+ T-cells obtained from draining lymph nodes following intravesical BCG administration. This action resulted in the production of TNFα and IL-2, cytokines found in patients receiving BCG therapy. Unfortunately, the investigators did not examine the cells for the production of IFNγ, a cytokine which our system indicates is pivotal for immune response.

The regulation of genes such as ICAM-1 may be controlled at a number of critical points. The transcription of genes appears to be tightly regulated, controlled by a superfluity of DNA binding proteins whose function is either to enhance or inhibit the activity of RNA polymerase II (Sen and Baltimore 1986). Splicing of immature RNA and poly-A tailing may also be rate limiting, however little is known of its control. The rate of transport of mature mRNA from the nucleus to the cytoplasm could present a restriction point. The stability of various mRNA species in the cytoplasm and the translation of mRNA into protein are among the best characterized mechanisms.

Induction of membrane ICAM-1 expression is a rapid event, detectable changes being evident within 4 hours of stimulation. However, with the exception of MGH-U1 there would not appear to be an intracellular pool of untranslated mRNA. For all the lines tested (except MGH-U1), actinomycin D pretreatment inhibited the induction of ICAM-1 expression due to IFNγ. Untranslated pools of mRNA have been described for a number of cytokines including IL-1, TNF and TGFβ (Beutler *et al* 1986a and b). The importance of such pools is that they can provide the potential for an extremely rapid response to external stimuli, particularly when the translation product is not stored in an intracellular vesicle. Only partial depression of ICAM-1 induction was achieved with MGH-U1, indicating the existence of cytoplasmic pools of untranslated mRNA or

protein.

Inhibitors of protein synthesis (such as cycloheximide, CHX) have been used to determine if protein synthesis is required for the induction of transcription (Fenton *et al* 1987). As expected, the cells which were sensitive to actinomycin D were also inhibited by CHX, thus indicating the requirement *de novo* mRNA and protein synthesis. In the case of MGH-U1 pretreatment with CHX failed to significantly depress ICAM-1 induction by IFNγ indicating the existence of intracellular pools of protein. Interestingly, later studies showed that PMA also induced ICAM-1 expression, this effect being inhibited by CHX to a greater extent than that of IFNγ. CHX would also appear to stabilize membrane associated ICAM-1 as treatment following IFNγ stimulation increases the percentage of cells expressing ICAM-1. The significance of these finding remains unclear, however the regulation of the genes for adhesion molecules is fundamental to the immune response and increased understanding of such mechanisms should lead to more comprehensive therapeutic strategies.

Following stimulation, ICAM-1 expression transiently increases. The fate of such ICAM-1 was investigated using ELISA able to detect soluble ICAM-1. The secretion of high levels of soluble ICAM-1 was noted, maximal levels correlating approximately with the decease in surface associated ICAM-1. Therefore, CHX may stabilize ICAM-1 by prevention of shedding. Furthermore, the links drawn between increased invasion of tumour and ICAM-1 expression (Tsujisaka *et al* 1991) may be in part due to the production of sICAM-1 rather than to the expression of ICAM-1 *per se*.

4.3 Functional evaluation of the role of adhesion molecules in LAK mediated cytotoxicity against bladder cancer

A number of lines of evidence suggest that in order for LAK cells to mediate their cytolytic effect, intimate liaison with the target cell is fundamental. Bladder cancer cell lines express two important cell adhesion molecules (ICAM-1 and ICAM-2), therefore a functional dissection of their role in LAK activity was undertaken.

Immunohistochemical data obtained from conjugates formed between LAK and TCC cells shows that the majority of LAK cells adhere to tumour cells which express ICAM-1. Those cells which fail to express ICAM-1 had significantly fewer LAK cells conjugated to them. Cell lines expressing the highest levels of ICAM-1 are more able to form conjugates than those which express less. This data indirectly implicates the ICAM-1/LFA-1 interaction in LAK activity.

Direct evidence for the role of ICAM-1 and ICAM-2 as target structures for primary recognition by LAK cells comes from adhesion blockade studies. Using the standard 4 hour cytotoxicity assay, the blockade of ICAM-1 (or ICAM-2) expressed by bladder cancer cells results in decreased susceptibility to LAK mediated killing, implicating these molecules as candidate ligands for LAK cells. The presence of antibodies to ICAM-1 and ICAM-2 throughout the duration of the assay results in greater inhibition of LAK activity than when used to pre-treat target cells. The reasons for this are unclear as treatment of LAK cells with anti-ICAM antibodies does not decrease their potency of killing. One possible explanation could involve the capping, internalization (and re-expression) or shedding of ICAM-1 molecules to which antibody has bound. It is also possible that following the interaction with LAK cells, the expression of ICAM-1 is induced on the target cell. This would not, however account for the result obtained with the ICAM-2 expressing cell line UMUC-3.

Blockade of LFA-1 (CD11a) on effector cells results in almost

complete abrogation of LAK activity (mean 83% depression). This levels of inhibition is greater than achieved by ICAM blockade on target cells. In one instance, MGH-U1, antibodies to LFA-2 caused a decrease in LAK activity. It is probable that ligands other than LFA-1 are important in LAK activity. Therefore, target lysis by LAK cells is critically dependent upon target binding properties. Several studies have addressed the question of adhesion molecules in NK and LAK activity. Quillet-Mary and colleagues (1991) demonstrated that binding of LAK cells to B-cell lines was essential for cytolysis, however LFA-1, LFA-3 and ICAM-1 were not the major ligands involved. This conclusion was reached following adhesion blockade and by a lack of correlation between expression and lysability, some of the premises under which the work presented in this thesis was carried out. However, a recent study has shown that in some cases the interaction of of LFA-1 with its ligand is necessary but not sufficient for its function (Dransfield *et al* 1992). Therefore, in addition to the initial LFA-1/ICAM-1 interaction, signalling events may dictate the functional consequences of adhesion. Rivoltini *et al* (1991) demonstrated that a drug resistant, epithelial colon carcinoma cell line was more sensitive to LAK activity than its drug sensitive counterpart. In addition, higher levels of adhesion molecules (including ICAM-1) were observed, which when blocked with antibodies resulted in a decrease in LAK mediated cytolysis. It is possible that the ligand for LAK cells will differ with different tumour cells.

The primary adhesion events involved in LAK activity against bladder cancer cells are almost entirely LFA-1 dependent, however a portion of these events are independent of ICAM-1 and ICAM-2. This data suggests the existence of a further ligand for LFA-1 expressed by bladder cancer cell lines. The existence of such a ligand on both NK cells and on T-cell lines has been suggested for some time (de Fougerolles *et al* 1991.

Akella and Hall 1992). Recently a third ligand for LFA-1 has been identified, characterized and termed ICAM-3 (de Fougerolles and Springer 1992). This ligand is highly expressed on lymphocytes, monocytes, and neutrophils, but is not expressed by endothelial cells. Its expression on epithelial cells remains to be determined but investigations are already underway.

Following the administration of intravesical BCG therapy high levels of IFNγ are detected in the urine. As discussed, this cytokine can rapidly induce and augment the expression of ICAM-1 on bladder cancer cells. The significance of such induction *in vivo* (in a manner similar to class II expression) remains to be determined. However, the function of increased ICAM-1 expression *in vitro* has been addressed. Cells stimulated with IFNγ are more susceptible to LAK mediated killing. Such increased lysis correlates well with increased ICAM-1 expression by the tumour cell. Furthermore, on cells which are not induced to express ICAM-1 (UMUC-3) no increase in cytolysis is observed. Similar observations have been made concerning the lysis of IFNγ treated neuroblastoma cells by LAK cells (Naganuma *et al* 1991). Stimulation with $100Uml^{-1}$ IFNγ resulted in increased ICAM-1 expression and increased cytolysis, which was preventable by antibodies to ICAM-1.

Therefore, it would appear that the lysis of tumour target cells is non-specific in that no specific antigen or antigen-recognition structures are involved. However, depending upon the tumour type (and possibly upon individual tumour cells) ICAM-1 would appear to serve as a primary recognition structure for LFA-1 bearing LAK cells. This would appear to be the case for bladder cancer cell lines. The function of LFA-1 and ICAM-1/-2 in LAK activity against bladder cancer cells is probably to maintain the cells in juxtaposition in order to permit delivery of a lethal hit. It is

unlikely that the adhesion interaction *per se* is responsible in the cytotoxic events which ensue.

Using a flow cytometric method the role of adhesion molecules in conjugate formation has been further dissected. It would appear that conjugation between LAK cells and bladder tumour cell lines is an extremely rapid event, occurring in less than one minute. Such conjugates once formed are very stable as repeated agitation and vigorous pipetting have no effect. The apparent high affinity of conjugation is well illustrated by scanning electron micrographs and immunohistochemical techniques which show an intimate relationship between the two cells. The differences in conjugation observed with the flow cytometric assay do not correlate with the differences in ICAM-1 expression or with the differential susceptibility of bladder cancer cells to LAK activity. In fact the least sensitive cell line (RT112) formed the most conjugates, suggesting events down-stream of primary adhesion are important in LAK mediated cytolysis. Whether or not the efficiency of target cell repair mechanisms has significant bearing on the sensitivity to LAK activity remains to be investigated. During situations of intimate contact signalling may occur between effector and target cells and *visa versa*. It is possible that cell lines which are less sensitive to LAK activity inactivate the effector cells thus inhibiting cytolysis. One explanation for the disparity between techniques lies in the number of effector cells per target, as the flow-cytometric method could not account for such differences whilst the microscopic evaluation could.

If the primary interaction between cells is mediated by the LFA-1/ICAM system then the expression of LFA-1 by LAK cells is mandatory but not sufficient to mediate adhesion. Rather, an active form of LFA-1 is required (Figdor *et al* 1990). Freshly isolated PBMC do not adhere to

bladder cancer cell lines. However, following activation with IL-2 a significant level of adhesion and cytotoxicity is observed. The adhesion of LAK cells to bladder cancer cells is critically dependent upon energy and the presence of certain divalent cations, in accordance with data for LFA-1 dependent functions (Dransfield *et al* 1989, 1990. Figdor *et al* 1990). Depravation of divalent cations from such cells prevents their forming stable conjugates with bladder cancer target cells. In the absence of Ca^{2+} a decrease in conjugation is observed whilst in the absence of all divalent cations almost total abrogation of conjugation is noted.

The studies of Dransfield have shown a role for three divalent cations in particular, namely calcium, magnesium and manganese (Dransfield *et al* 1992b). Such studies revealed the presence of a Mg^{2+} dependent epitope termed 24. Divalent Ca^{2+} ions are able to inhibit the expression of the 24 epitope and in parallel inhibit LFA-1 dependent T-cell binding. In the interaction between LAK and bladder cancer cells, Ca^{2+}, Mg^{2+} and Mn^{2+} are able to increase the formation of conjugates. Furthermore, in the absence of calcium ions both Mg^{2+} and Mn^{2+} increase conjugation to much higher levels. Calcium ions are able to compete with Mg^{2+} but not Mn^{2+} in the interaction between LAK and TCC cells. In contrast, the work of Dransfield showed that calcium could compete with both Mg^{2+} and Mn^{2+} for the expression of the 24 epitope and for T-cell adhesion. Dransfield's studies also showed that low binding occurred in the presence of Ca^{2+}, however high levels of binding are observed between LAK cells and TCC targets under similar conditions. These studies were found to function in a similar concentration range of cations (0.1-10mM). It has been suggested that Ca^{2+} may have a negative regulatory role in LFA-1 dependent functions (Dransfield *et al* 1992b). However, in the light of the evidence presented in this thesis, Ca^{2+} may not always be able to serve

such a function. Whether differences exist in the LFA-1 molecule on LAK cells as compared to classical T-cells remains to be investigated.

The increased binding of LAK cells treated with Mn^{2+} to bladder cancer cells is due to alterations in LFA-1 as both sodium azide and anti-LFA-1 antibodies were able to inhibit such functions. It is therefore probable that Mn^{2+} binds directly to one or more of the cation binding domains on the alpha chain of LFA-1 and in doing so causes a conformational change thereby increasing the affinity of LFA-1 for its ligands. Alternatively it is possible that the binding of cations to LFA-1 merely mimics the binding of LFA-1 to ICAM-1, imposing a similar conformational change.

Following binding of LAK cells to bladder cancer cells and the delivery of a lethal hit, de-adhesion is required for the LAK cell to again exert its cytolytic function. Presumably this would involve a conformational change. When de-adhesion is prevented (by treatment with mAb 24) LFA-1 dependent functions are inhibited (Dransfield *et al* 1992). The exact mechanisms of detachment are not known, whether divalent cations such as Ca^{2+} (possibly spilling from the target cell) could serve to switch LFA-1 to its "OFF" state remains unclear. However, a better understanding of this process is essential for a complete appreciation of the function of LFA-1.

4.4 The expression and function of interferon-gamma receptors

The expression of cytokine receptor is a prerequisite for the exertion of cytokine action, but is not sufficient for such. The results from Scatchard analysis indicate that high affinity IFNγ receptors are present in large numbers on the surface of four bladder cancer cell lines studied.

The number and affinity of IFNγ receptors on the Daudi cells in this study was in close agreement with the values found by other workers and would seem to verify the method of assay used here (Merlin *et al* 1985. K_d=0.5-5 x 10^{-10} and 3500-7000 receptors / cell). There were ten-fold fewer IFNγ receptors on the Daudi cells than on any of the bladder cancer cells ($p<0.01$). There was no statistical difference between the concentration of the receptors or their affinities amongst the four bladder cancer lines, and therefore the differential biological response of these lines to IFNγ could not be accounted for by the level of receptor expression. It would therefore seem that differences in biological response of individual tumour cell lines (and possibly tumours) to IFNγ are determined down-stream of the receptor-ligand interaction.

This study uses a competitive binding assay for the assessment of IFNγ expression. The results show a wide range of affinities and surface receptor numbers for the individual cell lines which has been the experience of other investigators using noncompetitive assay systems (Ucer *et al* 1985 and 1986). Ucer and colleagues found that receptor affinities on cells of the same line differed by a factor of ten at different times. Such differences have several possible explanations, both experimental and biological and could account for the large standard deviation of the results for IFNγ receptor expression on bladder cancer cells. The source and specific activities of the labelled IFNγ, as well as the labelling methods and the types of binding assays performed are important factors. The possible biological explanations for such variability, include tumour heterogeneity, alterations in receptor expression with cell cycle, the effect of increased cell density of receptor expression (Alexandrov *et al* manuscript submitted) and the presence of known modulating cytokines such as TNFα (Raitano 1990) and Granulocyte-Macrophage Colony

Stimulating factor (Fischer *et al* 1990).

Grups and Bange (1990) have examined the expression of Interferon-alpha receptors on three human bladder cancer cell lines (RT4, SD and 637V) and found no correlation between affinity and sensitivity to this cytokine. Jakse and colleagues (1988) have measured the expression of IFNγ receptors on two other human bladder cancer cell lines, 647V and J82, and found affinities of the same order of magnitude as those described here ($0.6\text{-}1.1 \times 10^{-10}$M). However, receptor numbers on the 647V and J82 cells were an order of magnitude lower (870-3000 receptors per cell). It would seem from this data that the expression of IFNγ receptors is not a useful parameter for predicting the response of bladder tumour cell lines to this cytokine and therefore would have no clinical use for determining response or prognosis.

It would appear that IFNγ binds to its receptor very rapidly, this event not being energy dependent as the kinetics of surface binding were similar at 37°C and 4°C. These results are substantiated by the earlier observations that only brief exposure of bladder cancer cells (<10 seconds) to IFNγ is required to generate significant biological response in terms of ICAM-1 expression. It is interesting that this is not the case for MHC class II expression by the same cells, suggesting that a higher threshold exists for biological effect (Hawkyard, personal communication). Following receptor binding IFNγ is internalized by the cell and here degraded, presumably after transportation into lysozomes. Although it is usual for the biological actions of IFNγ to be receptor mediated this need not necessarily be the case. Transfection studies have shown that cytoplasmic IFNγ can exert a variety of biological effects (Sanceau *et al* 1987). Whether internalized IFNγ can affect gene regulation via interaction with a potential nuclear receptor or whether alternative modes

of signal transduction exist inside the cytoplasm remains to be determined (Kushnaryov *et al* 1985). Nuclear receptors for IFNβ have been identified in various cells (Kushnaryov *et al* 1985 and 1986). Such nuclear receptors had a seven fold higher affinity for ligand than those in the plasma membrane. Microinjection studies have also elegantly demonstrated a possible intracellular role for IFNγ (Smith *et al* 1990b). In these studies human IFNγ was seen to induce Ia expression by murine macrophages, a phenomenon which was not due to membrane receptor binding as the actions of IFNγ are species specific (Raziuddin *et al* 1984). Other cytokines such as TNFα may also have intracellular actions (Smith *et al* 1990c). The function of intracellular IFNγ in the case of bladder cancer cells remains to be investigated, however it may account for the differential susceptibility of tumour cells to IFNγ.

4.5 The signal transduction of the interferon-gamma receptor

The exact mechanisms involved in signal transduction via the IFNγ receptor are unknown however, several investigators have attempted to delineate the primary events (Koide *et al* 1988. Dumont *et al* 1990. Nobukazu *et al* 1990. Celada *et al* 1991). It has been shown that PKC and calmodulin play crucial roles in the intracellular signalling of a large number of cytokines and growth factors (Isakov *et al* 1986. Means *et al* 1988). Earlier investigations have suggested that PKC and calmodulin were mediating the action of IFNγ in macrophages (Hamilton *et al* 1985. Celada *et al* 1986). The investigations within this thesis use bladder cancer cell lines and their response to IFNγ as a model for investigating the signal transduction pathway for this important cytokine. Three different cell lines were employed and the studies concentrated on two discrete

effects of IFNγ, namely the induction of MHC class II and ICAM-1 molecules.

Firstly the question of a role for PKC was addressed using PMA to activate PKC and the specific inhibitors H-7 and staurosporine. Differences are apparent between the cell lines in that PMA induces MHC class II expression on RT4 but not other cells and induces ICAM-1 expression on MGH-U1 but not on the other cell lines. The situation is further complicated by the lack of inhibition with H-7 and staurosporine. H7 inhibits PKC by competing with ATP, whilst staurosporine interacts directly with the catalytic domain of PKC specifically inhibiting its function. Furthermore, inhibition of this enzyme failed to prevent the induction of either MHC class II or ICAM-1 molecules on three cell lines indicating a lack of PKC in signal transduction. Activated PKC phosphorylates a range of cellular proteins by which a signal is transduced to the next steps (Nishizuka *et al* 1988). Therefore, in the case of PMA stimulation of RT4 and MGH-U1 it is possible that PKC activation mimicked a signal other than IFNγ. It is well known that phorbol esters, such as PMA, can activate PKC in a similar manner to diacylglycerol (DAG) (Nishizuka 1984). In order for PKC to become activated a rise in the concentration of intracellular calcium leads to binding of membrane associated PKC to Ca^{2+} (Huang 1989). No such increase in the levels of intracellular calcium is observed in bladder cancer cells following stimulation with IFNγ.

As initial studies suggested the involvement of a PKC independent pathway, the role of the Ca^{2+}/calmodulin pathway was investigated. The activation of the Ca/calmodulin-dependent protein kinase can be achieved by the use of calcium ionophores (Klee *et al* 1980). In contrast with other studies the calcium ionophores A23187 and ionomycin do not induce

ICAM-1 and MHC class II expression (with the exception of RT4) (Nobukazu *et al* 1990). Using the specific inhibitor of calmodulin, W7, the induction of class II molecules on RT112 is inhibited. However, the induced expression of MHC class II molecules on RT4 and MGH-U1 and the expression of ICAM-1 on any of the cell lines is not inhibited by W7. Removal of extracellular Ca^{2+} using EGTA resulted in a decreased MHC class II response of RT112 but failed to reduce the response in terms of ICAM-1 induction further suggesting differential usage of calcium ions for signal transduction pathways within the same cell type. The inhibition of intracellular calcium mobilization in RT112 also prevents the induction of class II antigens, this is not the case for RT4. Calcium channel blockade of RT112 also partially prevents the cell from expressing ICAM-1, similar blockade having no effect on either RT4 or MGH-U1 cells. In fact, the removal of extracellular calcium ions from these cells results in increased ICAM-1 expression. The reason for this remains unclear however, it is possible that calcium is in some way negatively regulating ICAM-1 expression in these cells.

The information from this area of investigation fails to provide a simplistic view of the signal transduction mechanisms for IFNγ in bladder tumour cell lines. Various lines of evidence implying a role for both PKC and Ca^{2+}/calmodulin dependent kinase. It would appear that different mechanisms are employed by the various cell lines and that the expression of MHC class II and ICAM-1 genes are further differentially regulated. It would be of interest to investigate the contribution of activated oncogenes to the signal transduction of the MHC and ICAM-1 gene products. It has been postulated that such effects due to oncogenes may occur at the level of MHC induction by interference with signal transduction (Morris 1990). Unfortunately the picture is further complicated by the work of others

suggesting signal transduction through cAMP (Gariglio *et al* 1988), calmodulin (Celada *et al* 1991), and PKC (Hamilton *et al* 1985. Yap *et al* 1986. Ostrowski *et al* 1988). Whether cells such as MGH-U1 express increased levels of oncogenes (such as *ras*) which inturn lead to decreased responsiveness in terms of MHC class II induction by IFNγ remains to be determined (Maudsley and Morris 1989).

4.6 The relationship of in vitro studies to the situation in vivo

In clinical immunology it is important for *in vitro* studies to have direct bearing and reference to the *in vivo* situation. For this reason several attempts have been made to link the *in vitro* findings and observations to the disease of superficial bladder cancer.

Previous workers have identified the presence of various cytokines in the urine following BCG therapy (Prescott *et al* 1990. Böhle *et al* 1990). In order to dissect the role of such molecules, the effects of recombinant versions have been investigated *in vitro* (Hawkyard *et al* 1991 and 1992). However, *in vivo* it is unlikely that a single cytokine is produced, in fact a cocktail of cytokines is usually active at any one instant. When urine from BCG treated patients is incubated with bladder cancer cells, phenotypic changes take place. The cells express MHC class II molecules and ICAM-1 molecules on their surface in much the same way as when treated with recombinant cytokines. These changes are not induced by urine from all instillations, the general trend being that with repeated instillation increased levels of potential biological activity are produced. The expression of MHC class II molecules occurs *in vivo* following repeated instillations of BCG (Prescott *et al* 1991). Therefore, it is possible that the same mediators in urine which induce phenotypic changes *in vitro* also do

so *in vivo*. Neutralizing antibody experiments demonstrate that a large proportion of the ICAM-1 inducing ability from patients urine is due to IFNγ. Using immunoassay techniques the presence of IFNγ in the urine samples was confirmed. As already mentioned, this is a point of debate. The work of de Boer (1991) does not confirm the presence of this cytokine in urine. One possible reason for this lies in methodological differences. In the study of de Boer urine samples were taken and immediately frozen. Prior to assay they were thawed, the cellular debris removed and dialyzed prior to re-freezing. The data presented in this thesis is derived from urine which was centrifuged and dialyzed to pH7.2 immediately, then frozen prior to determination of the cytokine content. Several problems exist within the method of de Boer. Firstly, the urine is stored at acidic pH. Studies from our group demonstrate that under such conditions IFNγ cannot be detected in urine as it is acid labile (S. Prescott, personal communication). Secondly, it would appear that repeated freeze-thaw cycles were used for urine. It is a generally held belief that cytokines are destroyed by such practices (A. Meager, personal communication). However, the same group have employed the methods recommended in this thesis and are still unable to confirm the presence of IFNγ (E de Boer, personal communication). Until independent workers confirm the existence of IFNγ in urine it will remain the subject of debate.

The cytokine TNFα is also produced following BCG treatment. However, it would not seem to have a major biological role in the induction of ICAM-1 expression. In fact, the synergy observed with recombinant cytokines is not observed between these cytokines in urine. One explanation could be that the TNFα in urine is immunoreactive but not bioactive. A further explanation for this could be due to the presence of soluble TNF-binding proteins. Such proteins are produced in normal

physiological urine, however their presence in the urine of BCG treated patients remains to be investigated (Englemann *et al* 1989). Therefore, for a clinical response to occur it may be that not only the level of cytokine produced is important but also the level of the soluble form of its receptor. Other soluble receptors are also produced under normal physiological conditions and secreted into urine including IL-2, IL-6 and IFNγ (Marcon *et al* 1988. Novivk *et al* 1989). In addition, the urine of patients receiving intravesical BCG therapy also contains elevated levels of soluble IL-2R (I. Milne, personal communication). The function of such receptors in normal urine is unclear. However, they may have an immunoregulatory role, either by participation in the process of eliminating cytokines via the kidney, or by modulating the availability of cytokines. Whether bladder cells or bladder tumour cells secrete soluble cytokine receptors also remains to be determined, however preliminary data suggests that *in vitro,* bladder cancer cell lines may secrete a soluble IFNγ binding protein of molecular weight approximately 75kDa (unpublished personal observation).

In addition to cytokines and cytokine binding proteins, soluble ICAM-1 is detected in the urine following BCG therapy, but not in normal urine. The levels of sICAM-1 increase with repeated instillation and can be detected within 12 hours of treatment. If the kinetics of ICAM-1 expression by cells *in vivo* are similar to those observed *in vitro* then maximal expression will not occur until 24 hours. Furthermore, the subsequent production of sICAM-1 molecules would not be maximal until after 72 hours. The source and function of such molecules is unclear. Studies have demonstrated that sICAM-1 molecules can exert powerful effects by inhibiting LFA-1 dependent functions *in vitro* (Becker *et al* 1991). Furthermore, in addition to its role as a cell adhesion molecule,

ICAM-1 has been identified as the receptor for rhinoviruses (Greve *et al* 1989. Staunton *et al* 1989). Furthermore, a soluble form of ICAM-1 inhibits rhinovirus infection (Marlin *et al* 1990). Whether other viral particles can be inhibited by this means provides an interesting avenue of research with potentially fruitful rewards. In bladder cancer patients, sICAM-1 could compete with membrane ICAM-1 for LFA-1 molecules on leucocytes, thus preventing immune recognition. Alternatively, sICAM-1 could act to promote transmembrane signalling via LFA-1 to the leucocyte as has been demonstrated (Van Seventer *et al* 1990. Wacholtz *et al* 1989).

In clinical practice, what is required is an accurate indicator of the tumour response to treatment. Failure to respond would allow a different modality to be employed. Whether sICAM-1 production by patients has any correlation with clinical response remains to be determined, however, unlike the presence of IL-1, IL-2, TNFα and IFNγ in the urine (which indicate an activated immune infiltrate) the presence of sICAM-1 may indicate a response of the tumour cells to these cytokines. This would seem to be the desired reaction. As shown *in vitro*, not all TCC cell lines respond to IFNγ in the same way, and *in vivo* a heterogeneous response is observed in terms of cytokine production and the secretion of sICAM-1.

4.7 A model for the action of intravesical BCG therapy for the treatment of superficial bladder cancer

The work presented in this thesis and that of other workers (namely Hawkyard, Prescott, Böhle and de Boer) combine together to give a picture of the immune response in BCG therapy (Figure 85). BCG organisms adhere to the tumour cells and are eventually endocytosed (Hawkyard,

personal observation). Whether various shock proteins such as heat shock protein (either human or bacterial) are released remains to be investigated, however such molecules could serve to activate the resident macrophages within the tumour. Following repeated instillation of BCG organisms a marked cellular infiltrate into the bladder wall and tumour occurs. This infiltrate contains immunologically active T-cells (both helper and cytotoxic), polymorphonuclear cells and macrophages. Such cells are the likely source of the cytokines which are transiently secreted in the urine of patients (IL-1, IL-2, IL-6, TNFα, IFNγ). These cytokines may serve two functions. Firstly they may further activate infiltrating cells and attract further circulating immunologically relevant cells to the site of inflammation. Such cells could then mediate the destruction of tumour cells by nonspecific cytotoxic mechanisms, as observed in the case of LAK cells killing bladder cancer cells *in vitro*. Secondly, the cytokines can exert direct effects on the tumour cells, inducing changes in the cell surface phenotype (increased MHC class I, class II, ICAM-1, and decreased EGF receptor as demonstrated by A. Alexandrov (manuscript in preparation)) and dramatically modify the growth rate of tumour cells (both cytostatic and cytotoxic mechanism being involved). Such phenotypic changes could alter the susceptibility of tumour cells to cytolysis be infiltrating cells, thus increasing the anti-tumour activity. Cells other than macrophages have been shown to act in an antigen presenting fashion. Whether tumour cells which express MHC class II can process mycobacterial antigen and express it in the context of class II molecules remains to be determined.

Figure 85. Possible mechanism of action of intravesical BCG

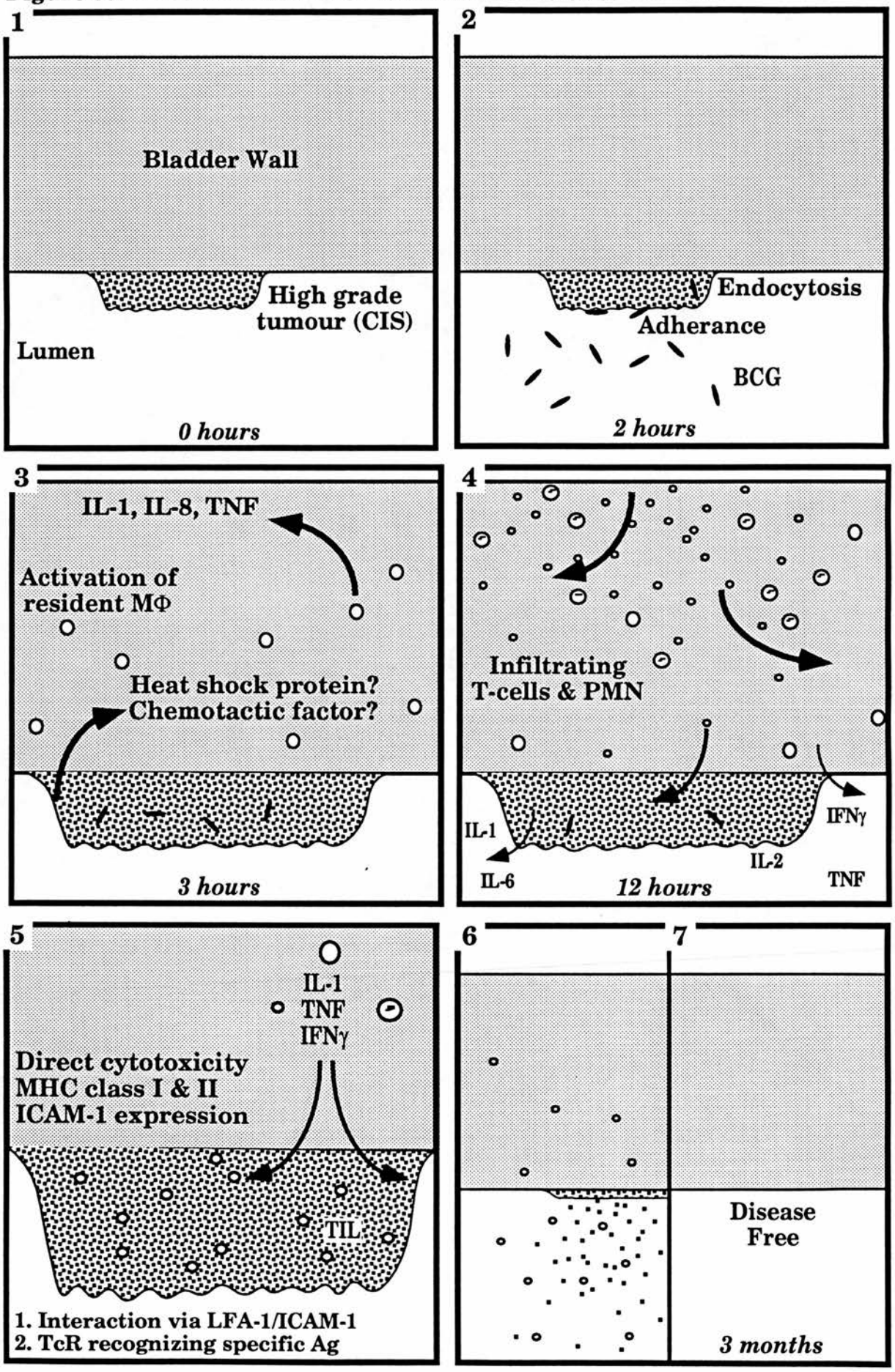


Figure 85. The possible mechanism of action of intravesical BCG

Shown is a possible series of events (1-7) in the successful adjuvant immunotherapy of superficial high grade bladder cancer using intravesically administered BCG organisms. This model is based on the studies of Prescott, Hawkyard, De Boer and on observations obtained from this thesis.

1. A high grade superficial bladder cancer.

2. BCG organisms are instilled into the bladder and allowed to interact with the bladder wall for 2 hours. Some organisms adhere to the bladder wall and tumour and are eventually endocytosed.

3. The interaction of BCG organisms with tumour cells, normal cells and resident macrophages leads to the release of heat shock proteins or chemotactic factors which activate the macrophages to produce inflammatory cytokines.

4. In response to the chemotactic and inflammatory signals, a large infiltrate of immunologically active cells occurs. These cells may be attracted to the site by changes in adhesion molecule expression in the local vasculature. Such infiltrating cells produce a variety of cytokines which are readily detected in the urine within 12 hours.

5. The cytokines produced by the infiltrate induce and augment the expression of MHC class I and II antigens and of ICAM-1 on the tumour cells. Such immunologically relevant molecules allow the interaction of tumour infiltrating lymphocytes (TIL) with tumour cells expressing ICAM-1. Furthermore, there is a possible role for specific BCG related antigen which may be presented by macrophages or tumour cells expressing MHC class I and II molecules to infiltrating T cells. In addition, IFNγ may exert direct cytostatic and cytotoxic effects on bladder cancer cells.

6. The tumour cells are sloughed off the urothelium into the urine and voided.

7. After 3 months the patient is examined and found to be disease free (approximately 80% of patients disease free at this stage).

Tumour cells - 
BCG organisms - /
Macrophages - O
T-cells - o
PMN's - ⊙
Dead tumour cells - 

These investigations have attempted to more fully understand the actions of LAK cells and BCG as used in the immunotherapy of bladder cancer. Although the findings, together with those of other workers, have gone some way to explaining the mechanisms of action, several key questions remain to be answered.

The source of cytokines and soluble ICAM-1 molecules in urine requires clarification. Although it is likely that helper T-cells are producing IFNγ, such speculation requires substantiation, possibly with the aid of *in situ* hybridization techniques. Preliminary investigations are now underway in our laboratory.

The function of increased ICAM-1 and MHC class II expression *in vivo* remains unclear. We are currently attempting to establish bladder cancer cell lines and autologous T-cell clones for use in antigen presenting studies. With such tools we aim to investigate the role of MHC class II molecules in possible antigen presentation to infiltrating T-cells. Such presentation would require specific antigen, the source of which could be either human (self heat shock proteins) or bacterial. The possibility of an auto-immune type response has been raised as the presence of auto-antibodies has been noted (Dr. A. Clarke, personal communication). Recently a report has emerged showing that murine bladder cancer cells are able to present antigen to BCG-specific CD4+ T-cells (Lattime *et al* 1992).

If the source of soluble ICAM-1 is tumour cells then studies of sICAM-1 production could provide a useful, easy-to-measure indicator of tumour response. Detailed studies into this possibility are already underway. Only with clinical response correlation can this parameter be

attributed with any merit.

The treatment regimen with BCG has been determined empirically, in Edinburgh six weekly instillations are given (complete response rate 70-80% at 3 months), in other groups instillations consisting of more frequent higher doses are given, yielding complete response rates in excess of 90% (D. Lamm, personal communication). If ICAM-1 expression has similar consequences *in vivo* as it does *in vitro* and behaves in a similar manner then the more frequent instillation protocols could prove more effective.

The mechanism of activation and adhesion of LFA-1 to its known ligands has been the subject of energetic investigations. Although not fully understood, a great deal is now known. Whether divalent cations actually bind to LFA-1 *in vivo* or simply mimic the interaction with ICAM-1 remains to be determined. However, little is known about the process of de-adhesion *ie* the detachment of LFA-1 from its ligand and its subsequent transformation to the "OFF" state. A more complete appreciation of the intricacies of LFA-1 function would be of great interest and could have clinical applications in chronic inflammatory disorders.

The clinical use of LAK cells has not yielded the results expected from initial studies *in vitro*. Furthermore, their use against bladder cancer has to date proved unsuccessful. It would appear that what is required is localized administration of high activated LAK cells. Ease of access to the bladder provides an opportunity to investigate such protocols for the treatment of invasive disease.

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Abbreviations

ACI	Adoptive cellular immunotherapy
ADCC	Antibody dependent cellular cytotoxicity
AFP	Alpha-foetoprotein
Ag	Antigen
APC	Antigen presenting cell
BCG	bacillus Calmette Guerin
BRM	Biological response modifiers
cAMP	cyclic adenosinemonophosphate
CD	Cluster designation
cDNA	Complementary Deoxyribonucleic acid
CEA	Carcinoembryonic antigen
CFDA-AM	Carboxyfluorsecein dacetate acetoxymethylester
CIS	Carcinoma *in situ*
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
DAB	Diaminobenzidine
DAG	Diacylglycerol
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetra acetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol aminoethyl ether tetraacetic acid
ELAM-1	Endothelial leucocyte adhesion molecule-1
ELISA	Enzyme linked immuno serological assay
Fc	Complement binding fragment
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GMCSF	Granulocyte macrophage colony stimulating factor
HBSS	Hanks balanced salt solution
HE	Hydroethidine
HLA	Huamn leucocyte antigen
HIV	Human immunodeficiency virus
HPV	Human pappiloma virus

HRP	Horse radish peroxidase
ICAM	Intercellular adhesion molecule
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-2R	Interleukin-2 receptor
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IFNα	Interferon-alpha
IFNβ	Interferon-beta
IFNγ	Interferon-gamma
LAK	Lymphokine-activated killer cell
LFA-1	Leucocyte function-associated antigen-1
MHC	Major histocompatibility antigen
MLR	Mixed lymphocyte reaction
mRNA	Messenger ribonucleic acid
M-VAC	Methotrexate, vinblastine, adrimycin, cisplatin
NCAM	Neural cell adhesion molecule
NK	Natural killer cell
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PGE	Prostaglandin E
PHA	Phytohaemagglutinin
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear cell
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
TAA	Tumour associated antigen
TBS	Tris buffered saline
TCC	Transitional cell carcinoma
TcR	T cell receptor
TGFβ	Transforming growth factor-beta

TIL	Tumour infiltrating lymphocyte
TNFα	Tumour necrosis factor-alpha
TNFβ	Tumour necrosis factor-beta
UK	United Kingdom
VCAM	Vascular cellular adhesion molecule
VLA	Very late activation-antigen

Appendix II

Amersham international plc
UK sales office
Lincoln Place
Green End
Aylesbury
Buckinghamshire

BDH Chemicals Ltd.
Burnfield Avenue
Thornliebank
Glasgow

Becton Dickinson UK Ltd.
Between Towns Road
Cowley
Oxfordshire

Behringwerke
Frankfurt
Germany

Boehringer Manheim Ltd
Bell Lane
Lewes
East Sussex

British Biotechnology Ltd
Watlington Road
Cowley
Oxford

Canberra Packard Ltd
Station Road
Pangbourne

Berks

Costar Corporation
One Alewife Centre
Cambridge
MA
USA

Coulter
Hialeah
Florida
USA

Dakopatts
22, The Arcade
The Octagon
High Wycombe
Buckinghamshire

Envair Ltd
Haslingden
Rossendale
Lancashire

Euro-Cetus B.V.
Amsterdam
Netherlands

Genzyme Diagnostics
Kingshill
West Malling
Kent

Gibco BRL Ltd
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Trident House

Renfrew Road
Paisley

LKB
232 Addington Road
Selsdon, South Croyden
Surrey

Molecular Probes
Junction City
USA

New England Nuclear Products Ltd.
c/o Dupont UK
Wedgewood Way
Stevenage
Hertfordshire

Pharmacia Ltd
Pharmacia House
Midsummer Boulevard
Milton Keynes

Phillips scientific
Cambridge
UK

Scottish Antibody Production Unit
Law Hospital
Carluke
Lanarkshire

Sera-Lab
Crawley Down
Sussex

Sigma Chemical Company
Fanct Road
Poole
Dorset

Skatron
Dolasletta 1
Tranby
3401 Lier
Norway

Sterelin Ltd
Lampton House
Lampton Road
Hounslow
Middlesex

T-cell Diagnostics, Inc.
Cambridge
MA
USA.